

Proton magnetic resonance studies of unsaturated and aromatic compounds with particular regard to effects of electron delocalization

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With 1 figure in the text

1. Introduction

In the very early papers dealing with nuclear magnetic resonance (NMR) of samples in the condensed states, Purcell *et al.* [14–16] and Bloch *et al.* [17, 18] pointed out that this new technique would not only provide precise values for nuclear gyro-magnetic ratios, but would also give information on the interactions between the nuclei and their environment, thus throwing light on the structure of solids and liquids. Their anticipations were realized when, shortly after, studies of nuclear relaxation and dipole–dipole interactions in solids provided multiform information on crystal structures, molecular motion and phase change in solids and molecular motions in liquids and gases (for review, cf. [19–21]).

Through the discovery of the chemical shift [22–24], it became clear that NMR could also be used for investigating the electronic structure of molecules. Improved resolution led to the observation of separate lines for chemically non-equivalent nuclei in the same molecule [24–26] and to the detection of isotropic nuclear spin–spin interactions [27, 28]. Hahn & Maxwell [28, 29] and Gutowsky, McCall & Slichter [27, 30] demonstrated that the observed high-resolution NMR-spectra could be derived from a phenomenological Hamiltonian of the form:

$$\mathcal{H} = \sum_i \nu_i I_z(i) + \sum_{i < j} J_{ij} \mathbf{I}(i) \cdot \mathbf{I}(j). \quad (1)$$

Here, $\mathbf{I}(i)$ and $I_z(i)$ are the operators for the total nuclear spin and its z -component respectively of particle i . The chemical shifts (ν_i) and spin-coupling constants (J_{ij}) are parameters which depend on the electronic structure of the molecule.

When studying high-resolution NMR [31–33], one is in general faced with the problem of (a) extracting numerical values of the NMR-parameters ν_i , J_{ij} from the experimental spectrum, and (b) relating the obtained values of these parameters to the molecular structure. For a better understanding of the relationship between NMR-parameters and electronic structure, systematic studies of closely related molecules are of great value. Conjugated and aromatic systems are particularly suit-

able in this respect, since the molecular structures of such compounds are in general well known and the theory of π -electron systems is a highly developed branch of molecular quantum mechanics.

In the present communication, a discussion of the phenomenological Hamiltonian (1) will be given first, followed by a review of some results of proton magnetic resonance studies of unsaturated and aromatic compounds, with particular regard to the effects of electron delocalization. Finally, some applications of NMR for structure determination of such compounds will be discussed.

2. The nuclear spin Hamiltonian

The evaluation of shifts and coupling constants from an experimental spectrum requires in general the exact or approximative diagonalization¹ of the NMR Hamiltonian (1). The simplest cases are encountered when the nuclei can be divided into groups (A, B, C, ...) such that all nuclei in a group are magnetically equivalent² and when the coupling constants between nuclei of different groups are much smaller than their relative chemical shifts. It has been shown [28-30, 43] that the square of the total spin angular momentum ($F^2(N)$) for a group N of magnetically equivalent nuclei is a good quantum number and that the mutual couplings of the spins within any one group of magnetically equivalent nuclei have no observable effects; consequently, the spin Hamiltonian (1) for a system composed exclusively of groups (A, B, C, ...) of magnetically equivalent nuclei may be simplified [30, 43] to

$$\mathcal{H} = \sum_N \nu_N F_z(N) + \sum_{N < M} J_{NM} \mathbf{F}(N) \cdot \mathbf{F}(M) \quad N, M = A, B, C, \dots, \quad (2)$$

where

$$F_z(N) = \sum_i^N I_z(i); \mathbf{F}(N) = \sum_i^N \mathbf{I}(i).$$

If all parameters are subject to the further condition: $|J_{NM}| \ll |\nu_N - \nu_M|$, $N, M = A, B, C, \dots$ the coupling terms are accurately handled by a first order perturbation calculation [30] and the Hamiltonian assumes the form

$$\mathcal{H} = \sum_N \nu_N F_z(N) + \sum_{N < M} J_{NM} F_z(N) F_z(M). \quad (3)$$

When this approximation is appropriate, the simple multiplet rules given by Gutowsky *et al.* [30] are applicable. When the coupling constants are no longer negligible as compared to the relative chemical shifts, the higher order perturbation expressions derived by Anderson [36] can be used to calculate the spectrum of (2), provided that none of the quantities $J_{NM}/[\nu_N - \nu_M]$ approaches one.

When a molecule contains chemically equivalent nuclei which are not magnetically equivalent, the secular equations may be factorized by the use of basic spin functions of appropriate symmetry [43, 44]. These results, together with a number of addi-

¹ However, cf. [34].

² A set of nuclei is said to be magnetically equivalent [35] when they have similar chemical shift and the same coupling constants to all nuclei of the other sets. The shorter term "equivalent" is more commonly encountered [6, 30, 34, 36-39] but might be misleading since it has been used [40, 41] to represent structurally (chemically) equivalent nuclei. The term similar (semblable) has been suggested by Abragam [37] for nuclei having the same chemical shift. The term "identical" [42] is synonymous with "magnetically equivalent".

tional rules for analyzing NMR-spectra, have recently been summarized in the monograph of Pople *et al.* [45] (cf. also [39]), and the following discussion will be confined to later results only.

The multiplicity of the spin functions for a group of magnetically equivalent nuclei remains unchanged during an allowed transition [28-30, 43] and therefore the spectrum of (2) consists of the superposition of the spectra obtained for the various combinations of "composite particles" (representing these groups) with fixed total spin. The spectra of complex spin systems contain [35, 39] explicit expressions for all transitions of the simpler systems which lead to the same combinations of group multiplicities. For example, the spectrum for a system consisting of two protons and one deuteron can be obtained [6] from the spectrum of an ABX_2 system¹ [47, 48] by neglecting all lines associated with the singlet state of the X_2 group; the simple AB spectrum is contained in that of an AB_3 system (cf. Tables 6-5 and 6-12 of [31]).

The matrix elements of the Hamiltonian (2) between basic spin functions, which may be chosen as eigenfunctions² of $F^2(N)$ and $F_z(N)$, can be evaluated by simple rules [41, 43, 45] applicable to the product functions. This is a rather tedious procedure, however, even if use is made of certain general regularities [49], and the matrix elements are more easily [35, 36, 42] evaluated by direct application of the general properties [50] of angular momentum operators without explicit reference to the form of the basic functions.

The basic functions mentioned above correspond to a representation characterized by the quantum numbers $F_z = \sum_N F_z(N)$; $F^2(N)$ and $F_z(N)$. It has been pointed out by Waugh & Dobbs [35] that in the general case of n interacting groups of magnetically equivalent nuclei, the number of off-diagonal matrix elements in this representation becomes $n(n-1)/2$ whereas the alternative representation characterized by the quantum numbers $F_z = \sum_N F_z(N)$; $F^2(N)$ and F^2 (where F^2 is defined by $\mathbf{F} = \sum_N \mathbf{F}(N)$) leads to only $(n-1)$ off-diagonal elements; consequently the latter scheme should be preferred in the analysis of spectra for systems containing more than two groups of magnetically equivalent nuclei. Some recent analyses of spectra for two interacting groups appear in [35, 39, 42, 51-53].

When the parameters of a group X of magnetically equivalent nuclei satisfy the conditions $|J_{NX}| \ll |\nu_N - \nu_X|$, N being any group other than X, enabling [38] the corresponding coupling terms to be changed to $J_{NX} F_z(N) F_z(X)$, one obtains spectra of the ABX [38, 41], ABX_2 [47, 48] and ABX_3 [54, 55] types. Recursive formulae for general ABX_n cases have been given [39].

It has been pointed out [6] that the matrix elements and the stationary energy levels of the nuclear spin Hamiltonian for any system containing only two strongly coupled nuclei of spin 1/2 can be obtained from a basic system characterized by the single quantum numbers $F_z(N)$. Examples of such spectra for systems containing four interacting groups have been analyzed by Hoffman & Gronowitz [6, 11].

Systems of several equivalent nuclei are generally encountered in molecules containing methyl or open-chain methylene groups, because the rapid internal rotation about the single bonds will usually (but not always [56-58]) establish the

¹ The well-known classification of spin systems due to Pople *et al.* [41, 46] is adopted in the following discussion.

² Tables of such functions for groups containing up to four nuclei are given in [39].

conditions for magnetic equivalence. Another commonly occurring class of spin systems is that in which all groups of magnetically equivalent nuclei contain only one particle of spin $1/2$. For example, the unsymmetrical three-spin system is encountered in vinyl compounds [59–63], in three-substituted benzenes [63–65] and in monosubstituted thiophenes [7, 66–68] and furans [68, 69]. Several properties of this system have been discussed¹ by Fessenden & Waugh [63]. They have found that the property of repeated spacings which exists in the weakly coupled, first order, case is preserved for any strength of the coupling. The observed splittings will provide good first approximations to the coupling constants even for quite strongly coupled systems [7, 63, 66], and are exceedingly useful for an assignment of the observed lines [7]. Relative signs of the coupling constants may be obtained directly from comparisons of relative observed intensities without any diagonalization of the Hamiltonian [63], a procedure used by Hoffman & Gronowitz [7] to demonstrate that the coupling constants in 2-substituted thiophenes are all of the same sign.

Since the theorems derived by Fessenden & Waugh [63] depend solely on some general properties [70] of unitary transformations similar rules obtain to more general spin systems as well [71].

The complete analysis of an unsymmetrical three-spin spectrum requires the diagonalization of 3×3 matrices, whereas the energy levels of an ABX system are obtained from 2×2 secular determinants and therefore can be conveniently written in closed form² [38, 41]. For this reason, it has been common practice to neglect off-diagonal elements involving not too strongly coupled nuclei, even when the neglected elements produce observable effects (for example, [65, 68]). Even though the calculated transition energies obtained in this approximation may differ from the observed line positions by less than the experimental errors, this does not verify the correctness of the NMR-parameters obtained [72]. To illustrate this further, let us consider a general ABC spectrum. Combination lines excluded, this spectrum shows four quartets assignable to the A, B and C spins with a multiplet structure determined by only three different splittings [63]. Therefore, the line *positions* are *exactly* reproduced in a first order perturbation calculation if the mid-points of the various bands are equated to the parameters ν_A , ν_B and ν_C and the coupling constants are adjusted to equal the observed splittings; clearly, the information obtained from the relative intensities cannot be disregarded.³

The ABX_n approximations may be improved upon by inclusion of the second order perturbation terms in J_{AX} and J_{BX} . This may be done either prior [47] or in succession [54, 71, 72] to the diagonalization with respect to J_{AB} . Reilly and Swalen have called their approach the ABK_n approximation [72]. If the nuclei A and B are nearly equivalent, a first approximation based on spin functions which are symmetric or antisymmetric in the nuclei A and B is preferable. This approach has been

¹ A similar treatment is given in [159].

² As has been pointed out earlier [6], this is a property common to all systems containing only two strongly coupled nuclei of spin $1/2$.

³ When estimating shift values, one might try to take the intensities into account by assigning to each line a weight proportional to its intensity. This simple procedure fails to give good shift values as the centre of gravity (ν''_N) and the midpoint (ν'_N) of any one band (N) fall on opposite sides of the theoretical shift (ν_N), both discrepancies being of similar orders of magnitude provided that the coupling is not too strong [7]. However, their average $f_N = \frac{1}{2}(\nu'_N + \nu''_N)$ is in general quite close to ν_N . This rule was extensively used in the analyses of the spectra of monosubstituted thiophenes [7].

called the AA'K approximation¹ [72]. Explicit formulae for ABK-, AA'K- and ABKY-spectra have been tabulated [71, 72].

When complete diagonalization of the Hamiltonian is required, two alternative approaches are feasible; either the various lines in the spectrum are first assigned to specified transitions² whereupon starting parameters for the calculation are derived from the lines so assigned [61, 63, 71-75], or starting values obtained in some other way are used as trial values in the nuclear spin Hamiltonian giving a calculated spectrum, which by comparison may provide assignments for the observed lines [4, 35, 42, 52, 53, 76]. In the first mentioned case, starting parameters can be obtained from exact formulae for some of the identified transitions [74, 75], by perturbational approaches [71, 72] by use of f_i -values [7] and by other methods [34]. When the lines cannot be assigned *ab initio*, the moment method [34] can still be used to derive some of the starting values from the observed spectrum [4, 42]. Information derived from structurally related, especially deuterated [4, 6, 77] or otherwise isotopically substituted [52, 53, 76], compounds has been found useful.

The values of the NMR-parameters are subsequently modified until the best over-all agreement between calculated and observed spectra is obtained. A trial and error procedure (even though capable of some systematization [11]) is rather cumbersome, especially for strongly coupled spin systems, and convergence is obtained more expediently if explicit expressions for the dependence of the observed line positions (or energy levels) on the NMR-parameters can be derived.

A simple approach to this problem was formulated by Hoffman & Gronowitz [4] in terms of perturbation theory [1, 4]. The idea is to obtain an accurate numerical solution to the eigenvalue problem of (1) with the starting values inserted for the NMR-parameters and then to treat the differences between the starting values and the correct values as a perturbation. A first order perturbation calculation gives linear equations relating the observed energies to the shifts and coupling constants. The calculations needed to obtain these linear equations are straightforward but somewhat lengthy. Essentially, this method uses the same unitary transformation to diagonalize the trial Hamiltonian and the full Hamiltonian. Less computational effort is required for the reverse procedure, in which the experimental (diagonal) energy matrix is transformed by means of the inverse transformation. This latter method is due to Reilly & Swalen [71, 72] and Alexander [73]. The perturbation approach has the advantage of being effective [4] even for spectra which are only partly assigned and it is readily extended to higher order terms. A comparative discussion of the two methods has been given [1, 78].

When the off-diagonal matrix elements are not too large, iterative methods based on second order perturbation treatments [79] may be applied.

As compared to methods based on the complete solution of the eigenvalue problem, the moment method of Anderson & McConnell [34] has the advantage of being applicable also to highly clustered spectra which cannot be analyzed in detail due to the large number of parameters involved (cf. for example [3]). A serious drawback is the impossibility of estimating errors in the values obtained, due to lack of knowledge of the contributions from far-away signals lost in the background. Primas

¹ This approach is of more general validity than the so-called ABB' approximation [64, 65] due to Richards & Schaefer [64], which leads to erroneous results unless the special conditions for negligible mixing between states of different symmetry in B and B' are fulfilled [4].

² Detailed considerations of the properties of the (diagonal) experimental energy matrix [63, 71-73, 159] are helpful in this procedure.

[80] has raised the fundamental objection to this method in that experimental and theoretical moments must be different, since the observed values include contributions from the shape-functions of the individual lines whereas the theoretical moments do not. In several cases, however, when the moment method has been compared with that involving a complete analysis of the spectrum [4, 42, 81] the differences were found to be small and of the order expected due to other [34] sources of error.

Another method of analyzing spectra without solving the eigenvalue problem of the Hamiltonian has been devised by Primas [80] and Primas & Günthard [82]. The theory of this method involves the Fourier transform (called the correlation function) of an idealized spectrum having lines of zero widths. The correlation function is used [80, 82] to derive a relationship between line intensities and transition energies of a general spectrum; it serves as generating function¹ for the spectral moments and can be used [80, 82] to obtain expressions corresponding to perturbation expansions. The presentation of the theory has been highly mathematical and it remains to be seen whether it will be much used for the practical analyses of NMR-spectra.

Shimizu & Fujiwara [84] have applied an interesting formal resemblance between the eigenvalue problem of (1) and that encountered in the classical problem of coupled harmonic oscillators. Although their approach offers no simplification in the arithmetics, it may be useful for instructive purposes. For example, one may see from the formulae pertaining to the ABX case [38, 41] that the band assigned to X will in general be a quartet even if one coupling constant—for example J_{BX} —is zero. This result is readily visualized in the mechanical case, where the strong coupling of A and B should lead to an “effective coupling” of B and X provided that X is coupled to A.

3. Chemical shifts and electron coupled nuclear spin-spin interactions

The phenomenological Hamiltonian (1) contains only nuclear spin operators. A complete theoretical description of the NMR experiment must involve the full Hamiltonian of the molecule, which in the customary fixed-nuclei approximation may be written:²

$$\mathcal{H}^{\text{tot}} = \mathcal{H}^{\text{e}} + \mathcal{H}^{\text{e}^{\text{i}}} + \mathcal{H}^{\text{en}} + \mathcal{H}^{\text{n}}. \quad (4)$$

$\mathcal{H}^{\text{e}}$ describes the molecule in the absence of external and nuclear magnetic fields and $\mathcal{H}^{\text{e}^{\text{i}}}$ represents the coupling of the electron spin and orbital moments with the external field. The term $\mathcal{H}^{\text{en}}$ represents the coupling of the nuclear magnets to the magnetic field from the electron cloud (including the Fermi contact [86] interaction). $\mathcal{H}^{\text{n}}$ contains in practice only the coupling of the nuclear spins with the external field, direct nuclear dipole-dipole couplings being averaged out by the rapid tumbling of the molecules.

Whereas the last term is ultimately responsible for the NMR phenomenon, $\mathcal{H}^{\text{en}}$ is the one which makes high-resolution NMR of interest in chemistry and molecular physics. Since the last three terms of (4) contribute little to the total energy of the

¹ The correlation function of Primas & Günthard corresponds to the characteristic function of a discrete probability distribution as defined in mathematical statistics [83].

² The present exposition is a slight modification of that given by McWeeny & Mizuno [85].

molecule, which is adequately calculated by means of perturbation theory, the effective nuclear spin Hamiltonian can be written

$$\mathcal{H}_{\text{eff}}^n = \mathcal{H}^n + \langle \psi_0 | \mathcal{H}^{\text{en}} | \psi_0 \rangle - 2 \times \sum_{k \neq 0} \frac{\langle \psi_0 | \mathcal{H}^{\text{en}} | \psi_k \rangle \langle \psi_k | \mathcal{H}^{\text{en}} | \psi_0 \rangle}{E_k - E_0} - \sum_{k \neq 0} \frac{\langle \psi_0 | \mathcal{H}^{\text{en}} | \psi_k \rangle \langle \psi_k | \mathcal{H}^{\text{en}} | \psi_0 \rangle}{E_k - E_0} + E_e. \quad (5)$$

E_e represents the the energy of the molecule in the absence of nuclear magnetism; $\mathcal{H}_{\text{eff}}^n - E_e$ constitutes the phenomenological Hamiltonian (1).

The perturbational derivation of (1) indicated by (5) is due to Ramsey [87–89] and Ramsey & Purcell [90]. A lucid presentation of their theories has been given by Abragam [37].

3.1 Chemical shifts

The parameter ν_i appearing in (1) may be written¹

$$\nu_i = \frac{\gamma_i}{2\pi} (1 - \sigma_i) H_0,$$

where H_0 is the external magnetic field, γ_i the gyromagnetic ratio of the nucleus i and σ_i its dimensionless screening constant. In Ramsey's formulation [37, 87, 88], the screening constant of an isolated diamagnetic molecule² is comprised of diamagnetic and paramagnetic contributions. The diamagnetic term corresponds [100] to the shielding provided by the Larmor precession of the electron cloud around the nucleus in question and may be evaluated if the ground state electronic wave function is known (for example, [101–103]). The second order paramagnetic term (which vanishes if the molecule is axially symmetric along the direction of the external field [104], arises from changes in the wave function caused by the perturbing field [105], and may be evaluated (in the Ramsey formulation) only if the excited electronic wave functions are known. This difficulty may be circumvented by the use of an "average excitation energy" [106] or by a variational treatment. Ramsey [87, 88] has shown that for linear molecules the paramagnetic term can be estimated from the spin-rotational interaction constant, which has been determined for a few molecules [107–109].

Although for simple molecules screening constants may be evaluated by the use of variational or perturbational calculations [101–103, 106, 110–120], in more complex molecules they can only be treated in qualitative or semiquantitative terms. This limitation arises not only from the lack of adequate wave functions but also from the partial cancellation of long-range diamagnetic and paramagnetic contributions [37, 121] resulting in the screening being represented by a small difference between two large quantities.

¹ Energies are given in frequency units.

² The influence of neighbouring molecules and of unpaired spins has been discussed by others [91–99].

For complex molecules, a subdivision of the screening constants into separate terms is customary. The different terms can be classified as [122]

- (a) intra-atomic diamagnetic contributions;
- (b) intra-atomic paramagnetic contributions;
- (c) contributions from other atoms;
- (d) contributions from inter-atomic currents.

This subdivision was first suggested by Saika & Slichter [123] and its justification has been discussed by Pople [124], McConnell [125], Stephen [112] and Ito [115]. A rigorous justification cannot be given since the resulting expressions are only approximately [125] gauge invariant. In semiquantitative applications of the above-mentioned division (for example, [8, 112, 125–127]), one is faced with the problem of choosing the proper origins, a somewhat arbitrary procedure [8, 125, 127].

The most clear-cut effect of electron delocalization on chemical shifts is the “anomalous” shift associated with aromatic compounds. Pople [128] suggested that this could be explained in the same way as the large diamagnetic anisotropy of these compounds, viz., by postulating [129] unhindered precession of the π -electrons around the molecular framework. It has been found that this simple model accounts qualitatively for both the relative shifts in condensed aromatic compounds [130–132] as well as for the diamagnetic screening of aliphatic protons confined to the vicinity of the hexagonal axis of an aromatic ring [131]. The minor discrepancies occurring have been ascribed to the insufficiency of the dipole approximation [115, 133], to the existence of paths other than those around a single ring [134], and to steric effects¹ (Reid [135]).

The concentration dependence of chemical shifts in mixtures containing an aromatic compound as a component has been explained [95, 138] in terms of the combined effects of the diamagnetic anisotropy and the disc-shape of such compounds. Formation of loose donor-acceptor complexes leads to an enhancement of the dilution shifts. Several proton magnetic resonance studies of such complexes have been published [138–143].

Hoffman & Kinell [2, 3] have tried to apply the ring current effect to structure determinations in their proton magnetic resonance studies of some poly-phenyls. The stable conformation of these molecules, especially of biphenyl (for references, see [3, 144]; cf. also [145]), has been the subject of a number of investigations. The results conclusively show that biphenyl is planar in the solid state, but the stable conformation in solution or in the vapour state has not been unambiguously determined. From most of the evidence available, it seems that biphenyl is best represented as a slightly hindered rotator [144, 146].

NMR-data could not provide additional information about biphenyl [3] because the variation of the ring current shifts for the various protons with dihedral angle is not considerably larger than the probable errors in the chemical shifts (which had to be obtained from spectral moments [34] or the estimated shifts originating from other causes. A nonplanar structure was inferred for *m*-terphenyl from its NMR-spectrum [2, 3]. This result is in harmony with the observation that 1,3,5-triphenyl benzene is not planar [147] even in the solid state [148]. The NMR-spectrum demonstrated that the perpendicular conformation observed for *o*-terphenyl in the vapour state [149] is also predominant in the liquid state and in solution [2, 3].

¹ Although Pople *et al.* [136] have criticized Reid's [135] neglect of conjugative contributions to the shifts, it is possible that the shift ascribed by them to conjugation [137] may be due in part to a neighbour anisotropy effect, which is already included [125] in the ring current model.

A similar study on the structure of stilbenes has recently been published by Katayama *et al.* [150].

Substituent effects on proton chemical shifts in aromatic compounds should correlate with the mesomeric effects of the substituents [151] and also with their inductive effects, provided that the local diamagnetic contributions to the screening (item *a* above) were dominant. Such relationships have been observed in the chemical shifts of some monosubstituted benzenes by Corio and Dailey [152] and others [138, 151–154]. However, the assumption that the change in intra-atomic screening entirely determines the shift is probably incorrect. Jackman [155, 156] has pointed out that the magnetic fields caused by the anisotropic susceptibilities of the substituents (item *c*) significantly affect the shifts of the *ortho* protons. Furthermore, the ring current shifts (item *d*) in the substituted compound may deviate from those in benzene, and (or) the neighbour anisotropy shift (item *c*) from the electrons on the ring-carbons are probably modified by the presence of the substituents. A parallelism between the two last-mentioned effects and the local diamagnetic screening would account for the observed interdependence of the chemical shifts and the inductive and mesomeric effects of the substituents.

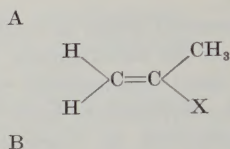
Recently, Buckingham [157] has suggested that the direct electrostatic field from the polar substituent may explain the shifts of monosubstituted benzenes. Although such an effect is expected, as was already pointed out by Stephen [92], it appears that the order of magnitude given by Buckingham is grossly overestimated. Following Buckingham [157], one would expect the shifts of the 4-hydrogens in 2-substituted thiophenes to be similar to those of the *para*-hydrogens in the corresponding benzenes, in obvious disagreement with the observed [8, 138, 152] data.

The most extensive investigation of substituent effects on proton chemical shifts in aromatic compounds has, to date, been undertaken by Gronowitz & Hoffman in their proton magnetic resonance studies of monosubstituted thiophenes [4, 6–8, 66]. They discussed the shift data mainly in terms of the inductive and mesomeric effects of the substituents; the contributions from the magnetic anisotropy of the substituents were also considered to some extent [8]. The general connection between the chemical shifts and the known electron-releasing or electron-attracting properties of the substituents have been pointed out, with the reservation that precise correlations are not to be expected [8] since chemical shifts are not determined exclusively by electron densities. The chemical shifts of hydrogen 5 in thiophenes substituted in the 2-position by a COX group were found [8] to follow Ingold's [158] mesomeric order. A similar ordering is evident in the chemical shifts of some 2-substituted propenes [156]. A comparison of the shifts of the hydrogens in *trans*-position to the substituent with those of the 5-hydrogen in related 2-substituted thiophenes is given in Table 1. The *trans*-hydrogens only were considered, since their shifts should be less dependent on the magnetic anisotropy of the 2-substituent [156].

As is evident from Table 1, the shift values of the thiophenes and the propenes show a similar dependence on the substituents (except for the halogen) but the shifts observed in the propenes are considerably larger than those in the thiophenes. The larger shifts in the propenes may be due to the fact that mesomeric charge displacement is confined to one resonance structure only in these compounds, whereas the charges in 2-substituted thiophenes are distributed [8] to the 3- and 5-positions and to some extent also to the sulphur or the 4-position.

The connection between the shifts in 2-substituted propenes and the mesomeric and inductive effects of the substituents was also mentioned by Whipple *et al.* [55].

Table 1. Chemical shifts of hydrogen A in some 2-substituted propenes [156]



relative to the shift in propene [241] and shifts of hydrogen 5 in some 2-substituted thiophenes [8] relative to the shift in thiophene.

Substituent X	Shift δ_A (ppm) in the 2-substituted propenes	Shift δ_5 (ppm) in the 2-substituted thiophenes
OCH ₃	(+ 1.1) ^a	+ 0.82
OCOCH ₃	+ 0.34	—
CH ₃	—	+ 0.28
CH ₂ C(CH ₃) ₃	+ 0.16 (+ 0.33) ^b	—
Cl	- 0.12	—
Br	- 0.57	+ 0.11
CONH ₂	- 0.41	—
COOCH ₃	- 0.53	- 0.20
COCH ₃	- 0.88	- 0.28
COCl	- 1.06	- 0.44
CHO	- 1.12	- 0.45 ^c
CN	- 0.80 (- 0.76) ^b	- 0.28 ^c

^a Value estimated on the basis of the difference between the shifts of 2-methoxypropene and 2-acetoxyprene (0.8 ppm [55]).

^b The assignments to *cis* and *trans* are uncertain [156].

^c The resonances of hydrogens 3 and 5 coincide [7, 8].

Banwell & Sheppard [159] have recently reported the proton magnetic resonance spectra of eight vinyl compounds. They found that the difference between the chemical shift of the =CH₂ group and that of the XHC= group is proportional to the Taft [160] resonance parameter σ_R , and they therefore concluded that inductive effects are less important. This conclusion does not necessarily follow, for the proportionality merely shows that inductive effects have a similar influence on the α - and β -carbons in a vinyl compound. Judging from the much larger range of shift values¹ found for hydrogens in the β -position relative to the substituent over that of the α -hydrogens, however, the inductive contributions to the shifts are smaller than the mesomeric ones. This conclusion is also suggested by the large high-field shift observed [159] for the *gem* ethylenic hydrogens in vinylfluoride. Greater importance of conjugative as compared to inductive contributions was also assumed by Taft *et al.* [154].

Some characteristic features of the thiophene chemical shifts [8] deserve mentioning, as they are of relevance in the current discussion (for review, see [161] on the participation of the sulphur *d*-orbitals in bond formation.

¹ Neighbour anisotropy effects being disregarded.

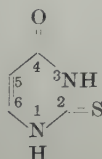
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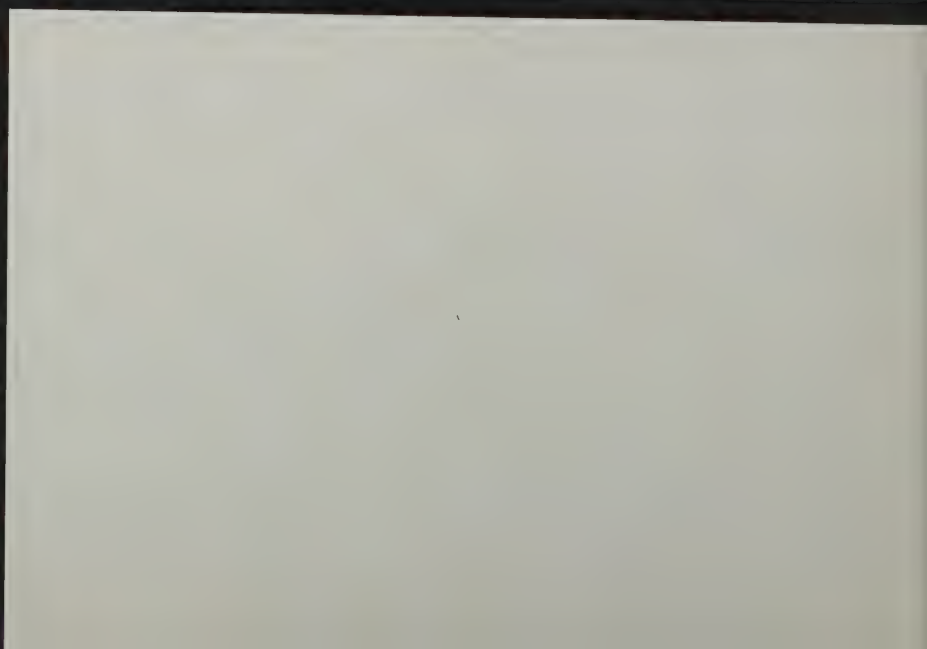
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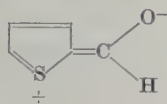
Formula XI on page 18 is erroneous. The correct formula should be



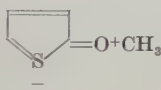


The claim that the sulphur *d*-orbitals ought to explain the physical and chemical properties of thiophene was first advanced by Schomaker & Pauling [162]. Following Longuet-Higgins [163], most quantum chemists [164–171] have regarded *pd*-hybridization an essential feature in the electronic structure of thiophene, and it has also been stated that the sulphur atom is to some extent equivalent to a $-\text{C}=\text{C}-$ group [163, 166–170]). De Heer questioned the generality of this similarity [164] and more recently some authors have even suggested that most of the properties of thiophene may be accounted for without invoking *pd*-hybridization at all [172, 173].

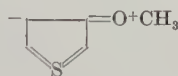
The anomalous behaviour of the 4-(*ortho*)-position in 3-substituted thiophenes has been ascribed to the lower weight of structures leading to decets on the sulphur [174] and of structures having $\text{C}=\text{S}$ double bonds [175]. The chemical shift data in [8] were discussed under the assumption that $\text{C}=\text{S}$ double bonds may reduce the weight of structures like I, and the occurrence of a decet on the sulphur will still further reduce the weight of structures like II.



I



II



III

On this assumption, it was possible to account for several of the characteristic differences between the shifts caused by electron attracting ($-M$) and electron releasing ($+M$) substituents [8]. That *d*-orbitals on the sulphur are still of some importance¹ may be inferred from the high-field shift of hydrogen 4 in thiophenes substituted in the 3-position with a $-I + M$ substituent. These shifts can be ascribed to the participation of resonance structures like III.

The low-field shift of hydrogen 5 in 2-thiocyanothiophene is also of interest in this context. The large value observed (-0.28 ppm) is of similar magnitude to those observed in 2-nitro-, 2-cyano- and 2-acetylthiophene and probably cannot be ascribed entirely to the inductive effect of the thiocyno group. However, the thiocyno group can attract electrons by mesomeric interaction only through expansion of the sulphur outer shell. A $-M$ effect of the thiocyno group has earlier been inferred from the dipole moments observed in the *para*-amino- and *para*-dimethylaminophenyl thiocyanates [176].

The study of isotropic proton resonance shifts in paramagnetics has recently attracted some interest as a tool for studying hyperfine couplings, the magnitudes and signs of which can be obtained in cases of unresolved electron spin resonance (ESR) hyperfine structure [177–183]. These investigations have provided experimental verification [177, 193] of the negative sign predicted [184] for the π -electron contact coupling of directly bound protons (fragment IV) and for the positive sign [182, 183] predicted [185] for the longer range couplings in fragment V.



IV



V

¹ The order of relative importance of the various resonance structures assumed by Gronowitz & Hoffman [8] is actually the same as that suggested by Schomaker & Pauling. These authors [162] consider that the classical structure should be given 70 % weight, structures involving $\text{C}=\text{S}$ double bonds and a positively charged sulphur were given 20 % weight and structures involving a decet on the sulphur were given a weight of 10 %.

With NMR measurements it is possible to arrive at definite assignments in favourable cases [182, 183, 186]. This is an additional advantage over the ESR method, where only the number of equally coupled nuclei can be determined.

It seems that this new field of study offers a potential method for investigations of the extent of conjugation in unsaturated and aromatic systems [186] and of electron delocalization in general. The interpretation of the observed shifts may be complicated in some cases due to isotropic paramagnetic shifts of origin other than contact coupling [98, 99, 187]; however, McConnell & Robertson [99] have shown that a separation of the effects is possible.

3.2 *Electron coupled nuclear spin-spin interactions*

Ramsey [89] has shown that the coupling constant $J_{NN'}$ between nuclei N and N' as given by perturbation theory (cf. equation 5) is composed of separate electron spin and electron orbital contributions, cross terms being negligible in the absence of electron spin-orbital coupling. The electron spin terms (which are obtained from the last sum in (5)) comprise the electron-nuclear dipole-dipole interactions and the contact coupling terms.

The orbital contributions to the coupling of nuclear spins in hydrogen deuteride constitute only a negligible fraction of the total coupling [89, 188, 189] and it has been stated that this result may be generalized to all proton-proton spin couplings [190, 191]. Pople [191, 192] has shown that the electron orbital contributions can be approximately evaluated from the anisotropy of the magnetic screening of the nuclei involved and the magnetic susceptibility of the other atoms in the molecule. By a specific example Pople demonstrated that the coupling via currents on a third atom is usually negligible. On the other hand, the subdivision into separate atomic contributions is not valid when induced orbital currents can occur along interatomic paths as in aromatic molecules [191] and it seems plausible that measurable orbital contributions to long-range proton spin couplings may exist in such molecules (cf. below). In general, one may safely assume that orbital contributions to proton spin-couplings are negligible, however, and most theoretical interpretations have been given in terms of the contact mechanism only [62, 194–206]. This mechanism produces indirect coupling of nuclear spins by admixture of the (singlet) ground state and excited triplet state wave functions. The perturbational calculation of contact coupled nuclear spin-spin interactions therefore involves the summation of an infinite number of terms. Variational calculations have as yet been applied only to hydrogen deuteride [116, 188, 189] and most theoretical interpretations have been based on the average excitation energy approximation. Although the applicability of this approximation to spin-coupling problems has recently been questioned [207], it will be assumed in the following that the results quoted are correct, even when derived by use of this approximation.¹

For directly bound nuclei the contribution of the electron-nuclear contact interaction to the nuclear spin couplings depends in part upon the degree of s -character in the bonding orbitals; thus, the observed couplings have been used as a criterion for orbital hybridization in chemical bonds [208–210].

The electron-nuclear contact coupling of proton spins N and N' in polyatomic molecules should vanish [200, 211] if the spins of the electrons around N and N' were entirely uncorrelated (as in the perfect pairing approximation [212]). The

¹ In certain cases a justification for this approximation can be given [202, 205].

existence of such couplings, therefore, is an indication of intrinsic delocalization of the bonding orbitals [211].

McConnell has shown [190] that the coupling of protons N and N' is proportional to the square of the bond order between atoms N and N' . According to Pople *et al.* [211] the coupling constant between protons N and N' , as given [190] by a one-determinant singlet wave function, is proportional to the excess of β -electrons over α -electrons at nucleus N , given that there is an electron of α -spin at nucleus N' . McWeeny & Mitzuno [85] have demonstrated that this is a general result for a singlet ground state, independent of orbital approximation. Hiroike has found [196–198] that the magnitude of the coupling $J_{NN'}$ is linearly related to the coefficients determining the weight of structures involving a long bond $N-N'$; likewise Karplus *et al.* [200, 203] have stated that the numerical value of the coupling constant between non-bonded protons is directly related to the deviations from perfect pairing.

From the preceding quotations it is evident that the study of proton spin-couplings will permit a critical evaluation of electron delocalization in organic molecules.

Karplus & Anderson [200] have found that the non-perfect pairing structures of methane, which contribute most to the proton spin-coupling, contribute only 0.056 eV to the binding energy of this molecule. It is interesting to note that the error introduced by the perfect pairing approximation has earlier [213, 214] been estimated to be of this order of magnitude (≈ 0.1 eV).

In saturated molecules, the coupling constants fall off very rapidly with the number of intervening bonds; an attenuation factor of 10 for each additional bond has been suggested [71, 194]. This result is to be expected, since structures involving a long bond should become increasingly unstable with increased separation of the formally bonded atoms. Longer range couplings may occur in conjugated systems where the perfect pairing approximation breaks down.

Some attempts have been made [9–11, 194, 195, 204, 205] to interpret the long-range couplings observed¹ in unsaturated and aromatic compounds in terms of a π -electron contact mechanism of similar kind to that used ([184, 185]; for further references see [221–223]) to explain the hyperfine structure in organic free radicals.

Using single configuration molecular orbital theory, McConnell [194] derived the expression

$$J_{NN'}^{\pi} = \beta^2 Q^2 \cdot \eta_{NN'}^2 / h \Delta E, \quad (6)$$

where β is the Bohr magneton, $\eta_{NN'}$ the π bond-order between carbon atoms N and N' and ΔE is the average excitation energy. The values of the *ortho*- and *meta*-coupling constants obtained from this expression were considerably smaller than the observed ones and it was concluded that these couplings are determined mainly by σ -electron contributions [194]. Valence bond calculations predict a negative sign for the π -electron contribution to *meta*-couplings [195, 224] and a positive sign for both the σ -electron [201] and the π -electron [195] contributions to the *ortho*-couplings.² Experimentally it has been found [217, 218] that the *ortho*- and the *meta*-couplings have the same sign; hence the *meta*-coupling must have the positive sign and consequently cannot be due to the π -electrons. This result provides further evidence for the correctness of the conclusions reached by the MO-calculation, viz. that the coup-

¹ For references, see [9, 11]. Cf. also [76, 156, 215–220].

² The sign convention is such as to give a lower coupling energy for antiparallel than for parallel nuclear spins.

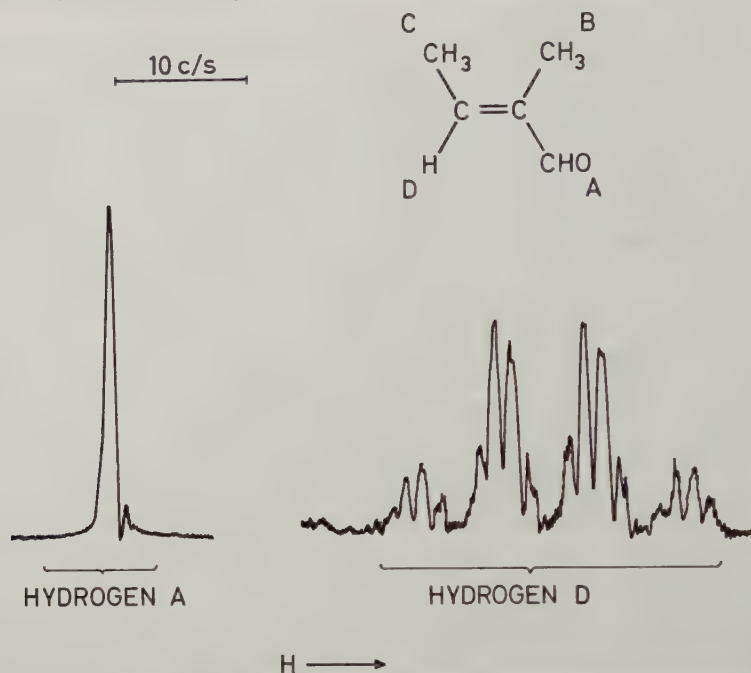


Fig. 1a. Aldehyde and ethylenic proton resonance bands of pure tiglaldehyde at 40 Mc/s. The aldehyde peak was recorded with reduced (1:5) gain.

The effects of second order perturbation terms [36], which are necessary for a complete analysis of the methyl group bands (cf. Fig. 1b), are unobservably small in this part of the spectrum, and one obtains on inspection; $\nu_A - \nu_D = 109$ c/s; $J_{CD} = 7.0$ c/s and $J_{BD} = 1.2$ c/s.

lings are dominated by σ -electron contributions. The *para*-coupling constants, on the other hand, may be dominated by the π -electron contributions [195].

The only negative¹ coupling reported between protons separated by a chain of conjugated (*not* hyperconjugated) carbon atoms is the one observed in a diene system [225]. It seems probable that the couplings observed between protons directly bound to unsaturated carbon atoms are often determined by mechanisms other than contact coupling combined with σ - π exchange interaction.

On the other hand, it has been found [9-11, 204, 205] that long-range couplings in systems, in which at least one of the hydrogens is attached to an atom bonded with a formal single bond to the unsaturated or aromatic system, are dominated by such interactions. The plausibility of this result is readily illustrated by application of equation (6), replacing the factor Q^2 by the product of the hyperfine splitting constants appropriate for directly bound protons (IV) and for methyl group protons (V) respectively. Theoretical [185] and experimental [182, 183] studies have demonstrated that these two constants are of similar magnitude but opposite signs. One is therefore led to the following predictions:

(a) The π -electron transmitted contact coupling of the proton spins should remain constant in magnitude [226] but change sign if a methyl group is substituted for a directly bound proton;

¹ The assumption is made that protons bound to vicinal carbons always have a positive sign as indicated by Karplus' calculations [201].

(b) The coupling over triple bonds should be twice¹ as large as those over double bonds [226].

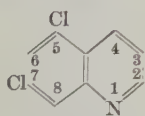
These predictions, which have been derived under the assumption of similar average excitation energies as well as equal hyperfine splitting constants for olefinic and acetylenic systems, are also arrived at by a more detailed theoretical treatment [204, 205].

The observed couplings have the magnitude and relative signs anticipated by the suggested π -electron coupling mechanism. Thus, the long-range coupling over one double and three single bonds in allylic systems (0.5–1.9 c/s) are invariably² negative (for references, see Table 3 in [11]); the long-range coupling over one double and four single bonds in 2-butenes (Table 4 in [11]) has a magnitude similar to the allyl group coupling and in tigraldehyde³ (Fig. 1) the sign is found to be positive.

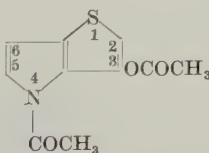
The long-range coupling in propynes (2.0–2.9 c/s, see Table 5 in [11] and also [76]) is about twice that in allyl groups (0.5–1.9 c/s); the long-range coupling in 1-butyne is of similar magnitude but opposite sign (Table 5 in [11]).

The coupling constants in allenes [227, 228] are also dominated by π -electron interactions, as suggested by Whipple *et al.* [227] and demonstrated by Karplus [204, 205].

The equality between the π -electron contact contributions to couplings of directly bound protons and those of methyl group protons can be used to estimate the contributions of the π -electron contact mechanism to couplings between directly bound protons in olefinic, acetylenic and aromatic systems [9, 11]. It has been shown [9] that the mutual coupling between the ring and the thiol protons in thiophenethiols is mainly due to π -electron contact coupling but that the side-chain couplings in thiophenealdehydes are largely determined by some other mechanism. This latter result is also indicated by the fact [5, 9] that the largest side-chain coupling in 3-thiophenealdehydes involves the 5-hydrogen; i.e. the aldehydic hydrogen in thiophenealdehydes [5, 9] and in furfural [5, 68, 69] couples to the α -hydrogen on the opposite side of the aromatic ring. "Cross-ring" couplings have also been observed in quinolines [219] such as 5,7-dichloroquinoline (VI), where $J_{48} = 0.8$ c/s, and in 3-acetoxy-4-acetylthieno [3,2-*b*] pyrrole (VII), where the coupling J_{25} was found [220] to have the value of 1.2 c/s.



VI



VII

The "cross-ring" coupling in quinoline probably cannot be explained in terms of a π -electron contact mechanism since the coupling $J_{CH,4}$ in 8-methylquinoline is

¹ The π_y and π_z contributions have to be considered separately [11].

² For the sake of brevity, the NMR-spectrum of tigraldehyde reported earlier [226] was said to be in agreement with an interpretation based on the assumption that all couplings have the same sign. Actually reversal of the sign of J_{ED} (cf. Fig. 1) does not change the calculated spectrum.

³ The NMR-spectrum was obtained with a Varian Associates model V-4300B, high-resolution spectrometer operating at 40.00 Mc/s and a flux-stabilized 12" magnet from the same company. The magnet sweep was calibrated with the modulation side-band technique using a 50.00 ± 0.05 c/s modulation frequency obtained from the power line.

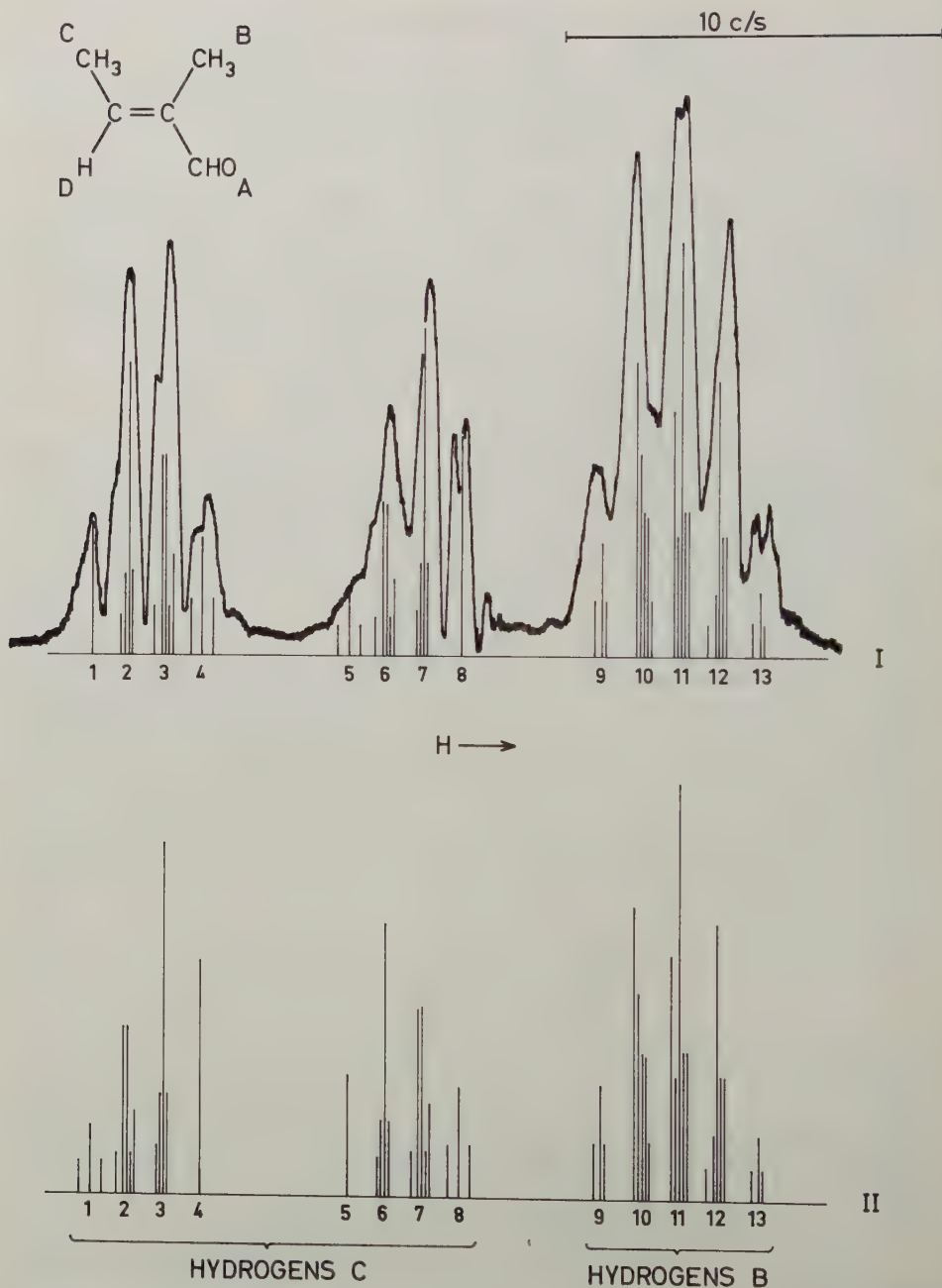


Fig. 1b. Methyl proton resonance bands of pure tiglaldehyde at 40 Mc/s. The magnetic field was swept from left to right at 0.55 ppm/minute. The apparent splitting of bands 8 and 13 and the shoulders on bands 3 and 4 are mainly due to the "wiggles" occurring after passage through intense (and comparatively narrow) peaks.

considerably less than 0.8 c/s as inferred from the structure of the 4-hydrogen band in the NMR-spectrum [219] of this compound.

It is tentatively suggested that all these "cross-ring" couplings are dominated by π -electron orbital contributions, because

(a) in the fused aromatic rings, the nuclear magnetic dipoles could give rise to induced π -electron currents having large magnetic moments;

(b) certain transitions involving the electrons of the C=O group are known [126, 229] to be associated with large paramagnetic circulations. The plausibility of this explanation cannot be assessed without some quantitative estimation of the orbital contributions, but one may note that the π -electron contact coupling of protons 4 and 8 in naphthalene is estimated [195] to have the value of 0.02 c/s, i.e. only 2.5% of the value observed for J_{48} in quinoline so the rejection of this mechanism seems reasonable.

4. Some applications of proton magnetic resonance to structure determinations

The characteristic shifts and coupling constants of different proton groupings [31–33, 230–232] makes NMR-spectroscopy a powerful tool for structure determination. When possible alternative structures of an unknown molecule differ with respect to the number of structurally equivalent hydrogens, the problem can be solved simply by measuring the number of bands and their relative intensities. In other cases it may be necessary to compare the shifts and coupling constants obtained with those observed in structurally related compounds, a procedure analogous to that followed in IR-spectroscopy.

An attractive feature of NMR-spectroscopy is the possibility frequently offered by this technique, of analyzing isomeric mixtures without previous isolation of the different components (cf., for example, Fig. 4 in [9]). This makes the NMR-method a very convenient one for studying tautomeric equilibria. A number of investigations have been published on keto-enol tautomerism in β -dicarbonyl compounds [233–236] and in β -triketones [237].

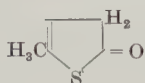
Tautomeric studies of heterocyclic compounds have been undertaken by Gronowitz & Hoffman [12, 13] and by Jones *et al.* [238]. NMR-spectroscopy has provided unequivocal evidence for the γ -thiolactone structure of 2-thienol and the so-called

In first order, the spectrum is expected to consist of a low field doublet of 1:3:3:1 quartets and (due to the near equality of J_{BC} and J_{BD}) a high field 1:4:6:4:1 quintet. The deviations from this simple pattern are caused by the superposition of the second order effects due to J_{BC} and J_{CD} . Spectrum (I) was obtained through a second order perturbation calculation [36] using the parameters $\nu_A = 307$ c/s; $\nu_B = 0$; $\nu_C = 10.8$ c/s; $\nu_D = 198$ c/s; $J_{AB} = J_{AC} = J_{AD} = 0$; $J_{BC} = \pm 1.0$ c/s; $|J_{BD}| = 1.2$ c/s; $J_{CD} = \pm 7.0$ c/s. For the lower spectrum (II) the same parameters were used except for the interchange of the relative signs for J_{BC} and J_{CD} . The calculated transition energies were rounded off to the nearest 0.1 c/s.

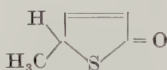
Comparing the observed spectrum with the two calculated ones, one arrives at the conclusion that spectrum II is incompatible with the observed spectrum; hence J_{BC} and J_{CD} must have like signs.

This conclusion is arrived at by the following considerations: (a) assignment II predicts a lower height of band 8 relative to band 4 because the former has a smaller (total) intensity as well as a greater line-width. Since in the observed spectrum band 8 is the higher, assignment II must be rejected; assignment I is in harmony with the relative heights observed; (b) the low height of band 6 can similarly be accounted for only with spectrum I; (c) the good resolution of bands 1 and 2 and the partial coalescence of bands 5 and 6 are in harmony with I, but are inconsistent with II, which predicts the reverse.

thiolenol [12]. The lower-boiling isomer of thiolenol [239] has been identified as 5-methyl-4-thiolen-2-one (VIII) and the higher-boiling isomer [240] as 5-methyl-3-thiolen-2-one (IX). Gronowitz & Hoffman have pointed out [12] that the earlier [240] structure assignments for thiolenol, which were based on chemical reaction data are erroneous.



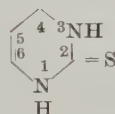
VIII



IX



X



XI

By comparing the NMR-data for 2-thienol with those of the two methyl derivatives they [12] concluded that 2-thienol exists as 3-thiolen-2-one (X). The correctness of these conclusions was confirmed by the IR-spectra of the three compounds. The isomerization of VIII to IX was followed in the NMR-spectra and the equilibrium composition determined.

The potentially tautomeric 2-aminopyrimidine, 2-hydroxypyrimidine and 2-thiouracil have recently been studied by NMR [13] in addition to previous extensive investigations by other physical methods (for references, see [13]). The NMR-spectrum indicated that in dimethyl sulphoxide solution the "thione-ketone" form (XI) predominates for 2-thiouracil, whereas the other two above-mentioned pyrimidines exist in the amino and hydroxy forms. NMR-spectroscopy shows that 2-aminopyrimidine exists in the amino form also in aqueous solution, whereas in 2-hydroxypyrimidine rapid protolytic exchange takes place, as indicated by the disappearance of the hydroxyl band and the appreciable broadening of the 4- and 6-hydrogen doublet lines in the spectrum of an aqueous solution of this latter compound. In the pyridone systems studied by Jones, Kartritzky & Lagowski [238], the protolytic exchange was so rapid as to preclude the observation of the tautomeric proton resonance, and the structure assignments were based on comparisons with spectra of suitably methylated derivatives.

In conclusion, it may be said that NMR-spectroscopy has proved to be a useful technique for studying tautomeric equilibria in open-chain as well as in heterocyclic systems, and is liable to become increasingly requested as a method of investigating equilibria in general in reversible processes.

SUMMARY

An exposition of some problems connected with proton magnetic resonance studies of unsaturated and aromatic compounds is given.

Following a critical discussion of some recent developments in methods of analyzing spectra, a review of various effects of electron delocalization on proton magnetic resonance spectra is given. This treatment includes the ring current effect in aromatic compounds, substituent effects on chemical shifts in unsaturated and aromatic compounds and isotropic contact shifts in paramagnetics.

The importance of electron delocalization in electron contact coupling of nuclear spins is pointed out and it is shown that certain long-range couplings in aromatic systems are not in agreement with predictions based on this mechanism.

Selected problems concerning structure determinations in unsaturated and aromatic compounds are discussed.

ACKNOWLEDGEMENTS

The author wishes to thank Prof. Kai Siegbahn for his interest and for the excellent facilities put at his disposal. Prof. Arne Fredga's interest in this work is likewise gratefully acknowledged.

Thanks are also due to Dr. S. Gronowitz and to Drs. T. Vänngård, L. Hedin and A. Fröman for many useful discussions. The author is indebted to Prof. M. Karplus, Prof. R. McWeeny and Dr. C. A. Reilly for making their as yet unpublished papers available to him, and to Dr. A. A. Bothner-By, Dr. W. D. Phillips and Dr. G. V. D. Tiers for communicating some unpublished results.

A grant from the *Swedish Technical Research Council* is gratefully acknowledged.

Institute of Physics, University of Uppsala, October 1960.

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Tryckt den 10 november 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB



Studies on carnosinase

III. Metal-ion stabilization of the enzyme*

By ANDREAS ROSENBERG

With 12 figures in the text

Introduction

In the course of the study of metal-ion activation of enzymes it is necessary to investigate the influence of metal ions on the stability of the enzyme molecule since it is possible that the observed activation is entirely due to the stabilizing effects of the metal ions in question [1]. In this case the catalytic process can proceed in the absence of the metal ions, although the process of denaturation may be so fast that it can be difficult to measure the catalytic process at all. Even in cases where it has been established that the metal ions play the role of a coenzyme, it is still possible that they likewise influence the stability of the enzyme molecule. Consequently the observed behaviour of the enzyme in the presence of metal ions can be a sum of metal-ion activation and stabilization. These two functions may be independent of each other, or the same metal ion, on the same site, can function in both capacities in close analogy to the well-known phenomenon of substrate stabilization of enzymes.

In the case of carnosinase of swine kidney Hanson and Smith [2], publishing the first observations of the behaviour of this enzyme, reported that the carnosinase activity in their preparations vanished when the solutions were not incubated with Mn^{++} ions. Later it was shown [3] that an efficient purification procedure for the enzyme could be devised when Mn^{++} ions were used as stabilizers. A study of metal-ion activation of carnosinase [4] established the fact that the metal ion participated as a coenzyme in the catalytic process. The rather complicated picture of activation could be explained in the simplest way by assuming that the metal ions also function in the capacity of stabilizers of the enzyme molecule. The results of activation studies indicated that the metal-ion specificity of these two functions was not necessarily the same.

The present investigation of inactivation and metal-ion stabilization has been planned as a part of the investigation into the mechanism of metal-ion activation. Thus stress has been laid on aspects connected with problems of the mechanism of enzyme action.

* Previous papers in this series, Ref. 3 and 4.

Materials and methods

Carnosinase

The enzyme was prepared from swine kidney as previously described [3]. The stock solution of purified enzyme containing 0.01 *M* MnCl_2 was kept at pH_5 7.75¹ in a refrigerator and its activity checked regularly.

Reagents

All solutions were made up with distilled and deionized water and stored in polyethylene bottles. The metal-ion solutions were prepared from analytical grade salts: chlorides in the case of Ca^{++} and Mn^{++} and sulfates in the case of Mg^{++} , Cd^{++} and Zn^{++} . Buffers were prepared from analytical grade salts, acids or bases. Sigma 121 tris(hydroxymethyl)aminomethane (THAM) was used for the preparations of THAM-HCl, THAM-maleate and THAM-acetate buffers. Solutions of ethylenediaminetetraacetic acid (EDTA) were prepared from commercial acid. The calculated amount of acid was dissolved by adding NaOH or THAM until the desired pH value was reached.

Substrate

Chromatographically pure L-carnosine from Th. Schuhardt A.G. was used as a substrate. The carnosine contained small amounts of heavy metal ions and was, as a rule, purified by dithizone extraction. The purification of solutions, water and glassware has been described previously [4].

Electrodialysis

The apparatus and the procedure were the same as described previously in connection with activation studies [4].

Measurement of carnosinase activity

The amount of carnosine hydrolyzed was determined by titrating the liberated NH_2 groups in 90 % alcohol. The details of the procedure and the method used for determining the endpoint have been described previously [4]. The concentration of the substrate was 0.05 *M* for all determinations. It should be emphasized that, unlike the results of the activity studies, most of the experimental points in this investigation do not represent one activity (moles of substrate split per minute) but a ratio between two activities, expressed as relative activity.

$$\text{Relative activity} = \frac{a \times 100}{a_0}$$

a_0 represents the initial activity and a the activity after a chosen period of inactivation. This means that, since the relatively large errors in individual activity measurements are mainly due to differences in the volume of aliquots and also to differences in the volume of the components of the reaction mixture [4], the dispersion of the

¹ The subscript indicates the temperature of the pH measurements.

experimental values increases considerably. In order to minimize this tendency, the volumetric measurements with pipettes had to be performed with meticulous care. The same pipettes were used for the same measurement within each series. The pipettes were cleaned between each individual measurement with a mixture of concentrated sulfuric and nitric acids (1:1), washed with deionized water and alcohol, and then dried.

Experimental results

Kinetics of inactivation

In this context the term inactivation is used synonymously with irreversible inactivation. Percentage of inactivated protein is defined as the measured percentage of irreversible loss of activity of the enzyme when measured with carnosine as substrate.

The first series of measurements shows the inactivation of the enzyme when the stabilizing Mn^{++} ions were removed. The inactivation was studied at different temperatures and at constant pH. The buffer used was THAM-HCl of constant ionic strength ($\mu = 0.1$). The pH was adjusted to 7.65 at different temperatures by adjusting the amount of THAM in the buffer. The Mn^{++} ions were removed by addition of a solution of EDTA-Na of pH₂₅ 7.65. The experiments were performed as follows. The concentrated enzyme solution containing 0.01 *M* Mn^{++} was diluted with buffer so that the Mn^{++} concentration was 0.001 *M*. The temperature of the diluted solution was brought to the desired point and enough of the concentrated EDTA solution was added to bring the EDTA concentration in the enzyme solution to 0.002 *M*. 100 μ l aliquots were removed at indicated time intervals and mixed with an equal volume of 0.02 *M* $MnCl_2$. The activity of the aliquot was then determined at pH₄₀ 7.65 and at 40°. Fig. 1 represents the loss of activity as a function of time at different temperatures. Due to the addition of EDTA in the form of Na^+ salt a constant amount of Na^+ ions was present in all the experiments. When the experiments were repeated at 30° and 40° with the use of EDTA-THAM, the influence of the presence of Na^+ ions turned out to be of the same order as the dispersion of the experimental points. At lower temperatures, however, a certain effect could be distinguished. Fig. 2 shows the inactivation at 11° for 4 different procedures for removal of Mn^{++} ions; two of them in the presence of sodium ions and two without any metal ions present. In order to ascertain that the changes in stability are not very sensitive to small changes in ionic strength, the inactivation at 40° was measured after the addition of extra NaCl. No significant change in the rate of denaturation was observed with 0.015 *M* extra NaCl present. In Fig. 3 the time course of inactivation has been plotted on a semilogarithmic scale.

The chemical nature of inactivation

The following experiments were carried out for the purpose of elucidating the chemical nature of inactivation. In order to determine whether a proteolytic degradation of the protein takes place during the process of inactivation, the increase in ninhydrin colour was measured in a sample of enzyme solution. A part of the concentrated enzyme solution was electrodialed for this purpose. Samples were removed at intervals from the metal-free enzyme solution kept at 40° and pH 7.65 and the increase in ninhydrin colour determined, first in a part of the intact sample,

and then after the removal of protein material by precipitation with trichloroacetic acid. The increase of ninhydrin colour in both cases was within the limits of error, which at most corresponds to the liberation of 2 to 3 leucine equivalents per enzyme molecule. The molecular weight of the protein was assumed for this purpose to be approximately 50,000.

Paper chromatography

50 μ l of the concentrated enzyme solution was mixed with 25 μ l of 0.1 *M* EDTA-Na and kept at 40°C for 1 hour. The solution soon became turbid, and a considerable precipitate of protein had been formed after 60 minutes. 50 μ l of the suspension (containing approximately 0.15 mg protein per ml) was applied to the paper together with an identical sample of the stable enzyme solution. Acetic acid-*n*-butanol-water (1:4:1) was used for an ascending chromatogram. Both samples showed an equal and very faint trace of free amino acids.

Paper electrophoresis

A part of the concentrated stock solution of the enzyme was inactivated as described for paper chromatography. A sample of the suspension and an equal amount of the original and unaltered solution were applied to Whatman 1 filter-paper. Veronal buffer of pH₂₀ 8.6 containing 0.001 *M* Ca⁺⁺ was used for the electrophoretic run. Staining with bromophenol blue revealed that the bulk of the protein material of the inactivated sample had remained immobile at the point of application, whereas the only other visible component had the same mobility as the intact enzyme.

Attempts to use the ultracentrifuge for analysis of solutions of the inactivated enzyme have hitherto been unsuccessful due to the precipitation of inactivated enzyme from concentrated solutions.

The time course of reactivation by Mn⁺⁺ ions

The next experiments were devised to elucidate the time course of the reactivation or more precisely the existence of a lag period in activity after the addition of Mn⁺⁺ ions to the metal-free enzyme solution. A part of the stock enzyme solution was electrodialedyzed; meanwhile a mixture of THAM-HCl buffer of pH₄₀ 7.65 and the substrate solution was brought to the temperature of the assay. Then a suitable amount of the freshly dialyzed enzyme solution was added and the reaction vessel with the solution vigorously shaken. After some minutes when the temperature of the solution was stabilized, a small volume of prewarmed, concentrated MnCl₂ solution was added, so that the final Mn⁺⁺ concentration was 5×10^{-3} *M*. The moment of Mn⁺⁺ addition was assumed to be the zero point of the hydrolytic reaction. Such assays were carried out at 25°, 30° and 40°. The experiments were then repeated with the difference that the metal-free enzyme solution was allowed to stand at 10° for up to 30 hours after the end of the electrodialedysis and before the start of the assay. The time course of the reaction was in all cases represented by straight lines. Three representative runs are shown in Fig. 4. Due to the time-consuming manual procedure for the withdrawal of samples and for the titration, no points could be determined previous to 5 minutes. The point at 5 minutes is subject to a much larger error than the following points. Thus nothing can be said about a small lag period during the very first minutes.

Stabilization of the enzyme by Mn^{++} , Ca^{++} and Mg^{++}

The influence of these metal ions on the stability of the enzyme was determined at pH_{40} 7.65 and at 40° . The total concentration of the stabilizing ions was in each case $5 \times 10^{-3} M$. The electro dialyzed metal-ion free enzyme solution was added to a prewarmed buffer solution containing the stabilizing metal ions. At indicated time intervals aliquots were withdrawn from the solution and pipetted into a buffer solution containing Mn^{++} . Substrate was added and activity determined under standard conditions at 40° . The total Mn^{++} concentration in the assay was $2 \times 10^{-3} M$. The results are shown in Fig. 5, where the inactivation is expressed as relative activity. The stabilization by Mn^{++} ions was also investigated at lower and higher pH values than 7.65. Fig. 6 represents the inactivation as a function of time at pH_{40} 6.0 and at 40° in the presence of $0.01 M MnCl_2$. A part of the stock enzyme solution of pH 7.70 containing $0.01 M Mn^{++}$ was mixed for this purpose with an equal volume of Na acetate-acetic acid buffer containing $0.01 M MnCl_2$. The concentration and pH of the acetate buffer was chosen so that the pH_{40} of the resulting mixture was 6.0. The loss of activity was determined by withdrawing aliquots, the activities of which were determined at pH_{40} 7.65 and at 40° under standard conditions. The stability in the presence of $0.01 M Mn^{++}$ at higher pH values is represented by Fig. 7. The experiments shown were carried out in the same fashion as described for the experiment at pH 6, with the exception that THAM buffers of suitable pH were used instead of acetate buffer. The inactivation at higher pH values was always accompanied by precipitation of MnO_2 .

The stabilization of the enzyme by Cd^{++} and Zn^{++}

Since both the concentration of the stabilizing ions and the pH of the experiments was different from those of the preceding measurements, the results with Zn^{++} and Cd^{++} are presented separately. The experiments with Zn^{++} and Cd^{++} were carried out at pH_{40} 7.0 in order to avoid the precipitation of $Zn(OH)_2$. The total concentration of Zn^{++} was $1 \times 10^{-3} M$ in all experiments. Higher concentrations of Zn^{++} resulted in visible precipitation of protein directly after the addition of Zn^{++} . A tendency to form precipitates was also observed, when higher concentrations of Cd^{++} ions were used. Even at a lower Zn^{++} or Cd^{++} concentration than $1 \times 10^{-3} M$ the inactivation was followed by a growing turbidity of the solution. Experiments with Cd^{++} ions were carried out with several different concentrations as indicated in Fig. 8. Complementary to these measurements the stability was also measured at pH_{40} 7.65 with $5 \times 10^{-3} M Cd^{++}$ as the stabilizing ion. The inactivation in this case followed approximately the same course as at pH 7.0.

The experiments were carried out as follows. The stabilizing metal ions were added to a THAM-maleate buffer of pH_{40} 6.95. The solution was kept at 40° for 1 hour to ascertain that neither precipitate nor turbidity appeared. After that a part of the freshly dialyzed enzyme solution was added and the pH of the mixture measured. Aliquots were now withdrawn at indicated time intervals. When Zn^{++} was used as the stabilizing ion, the activity of the aliquots was measured in the presence of Mn^{++} ions in order to avoid extensive inactivation during the activity measurement. In spite of the inhibiting effect of Zn^{++} ions the activity after addition of Mn^{++} ions was considerably higher than in the presence of Zn^{++} ions alone. The total Mn^{++} concentration in the assay was in this case $5 \times 10^{-3} M$. The activity in the presence

of Cd^{++} ions was so high, that the activity measurements were carried out in the presence of Cd^{++} ions only. Extra Cd^{++} ions were added to the assay to compensate for the ions bound by the substrate. The final total concentration of Cd^{++} was $2 \times 10^{-3} M$. The pH of the activity measurements was pH 7.65. There was no precipitation of $\text{Zn}(\text{OH})_2$ as most of the ions were removed by chelate formation with the substrate. The use of Cd^{++} ions as activators implies that the activity measured both at zero time and at any other time included the inactivation during the 20-minute period of activity determination, the activity in this case being expressed as moles of carnosine split during the first 20 minutes.

Stability as a function of pH and Me^{++}

Experiments to determine the stability as a function of pH were performed in the presence of Mn^{++} , Cd^{++} and with the metal-free enzyme. For this purpose a number of THAM-HCl, THAM-maleate, acetate and borate buffers were prepared, electro-dialyzed enzyme added, and the pH_{40} determined. In the case of Mn^{++} and Cd^{++} stabilization, metal ions were added so that the total Mn^{++} and Cd^{++} concentrations were $5 \times 10^{-3} M$ and $1 \times 10^{-3} M$ respectively. The activity was measured directly and after 1 hour at 40° . The activity measurements were carried out in the presence of $2 \times 10^{-3} M$ Mn^{++} , except in the case of Cd^{++} stabilization, where Cd^{++} ions were used in the total concentration of $2 \times 10^{-3} M$. Fig. 9 shows the relative activity after 1 hour at 40° as a function of the pH of the enzyme solution.

Fig. 10 represents the relative activity as a function of the concentration of the stabilizing metal ion after 1 hour at 40° and at pH 7.65. The activity was measured at pH 7.65 and at 40° after addition of Mn^{++} so that the total Mn^{++} concentration in the assay was $2 \times 10^{-3} M$. The metal-ion concentrations indicated are the total metal-ion concentrations and not, as in the case of activity studies [4], the free metal-ion concentrations. This is done because the Mn^{++} binding to THAM is so low [5], that the determinations of free Mn^{++} concentration are rather uncertain. In any case a 10 to 20 % difference in the metal-ion concentration is of little consequence to the argument. The same is assumed to be the case for Ca^{++} ions, where preliminary measurements with the method of Raaflaub [6] indicated that the binding constant to THAM is still lower than the constant for Mn^{++} .

Inhibition of Mn^{++} activation by Ca^{++}

The inhibition was studied at a constant Mn^{++} concentration by varying the amount of Ca^{++} present; this was done in order to eliminate the effect of varying Mn^{++} concentration on the stability of the enzyme. One part of the stock enzyme solution containing 0.01 M Mn^{++} was transferred into a dialysis bag and equilibrated against two 500 ml volumes of THAM-HCl buffer pH_5 7.70 containing $3 \times 10^{-3} M$ MnCl_2 ; another part of the enzyme solution was treated identically, but the buffer solution used for equilibrating contained $6 \times 10^{-3} M$ MnCl_2 . The activity of these enzyme solutions was then determined at pH 7.65 and at 40° in the presence of varying amounts of Ca^{++} added in the form of CaCl_2 solution. The results presented in Fig. 11 are expressed as a plot of v_0/v against $[\text{Ca}^{++}]$ as proposed by Laidler [7].

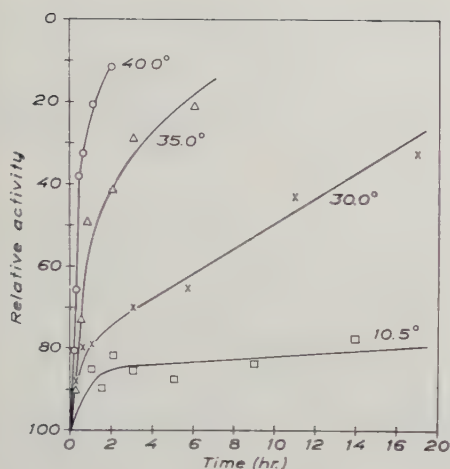


Fig. 1.

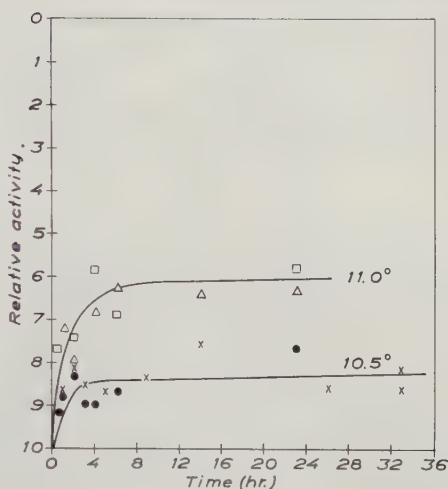


Fig. 2.

Fig. 1. The irreversible inactivation of metal-ion-free carnosinase as a function of time after an excess of EDTA-Na had been added at $t = 0$.

Fig. 2. The irreversible inactivation of metal-ion-free carnosinase at 10.5° and 11.0° . $\square - \square - \square$ after the addition of EDTA-THAM, $\triangle - \triangle - \triangle$ after electro dialysis, $\times - \times - \times$ after the addition of EDTA-Na, $\bullet - \bullet - \bullet$ after electro dialysis in presence of 0.015 M NaCl .

Discussion

The first question to be discussed is the nature of the process of irreversible inactivation of carnosinase when the divalent metal ions present are removed by chelating agents or electro dialysis. The disappearance of activity may be due to an attack by traces of proteolytic enzymes present in the preparation of carnosinase, the susceptibility of carnosinase to the attack being reduced by the presence of divalent ions. In this case the enzyme can be stable also in the absence of metal ions if the traces of proteolytic enzymes are removed as already described in the case of α -amylases [8]. If, on the other hand, the role of the divalent ions is to stabilize an otherwise labile protein conformation, the removal of the ions ought to be followed by thermal denaturation of the protein, operationally defined by changes in viscosity, solubility and optical rotation in the absence of proteolytic breakdown of the protein chain. The experiments presented show that the inactivation of carnosinase is not followed by any significant increase in ninhydrin colour. No increase in free amino-acids is shown by paper chromatography nor is the presence of any larger peptide fragment indicated by paper electrophoresis. This indicates, that inactivation due to proteolytic breakdown is very improbable.

The preparation of a concentrated solution of the inactive enzyme to use in the ultracentrifuge failed, due to the precipitation of the enzyme. This is at the same time proof of thermal denaturation of the enzyme after inactivation, as decreased solubility is one of the most common and general properties of thermal denaturation as defined above. Precipitation in itself is naturally not adequate as a sole criterion of the denaturation. It is necessary to show that the kinetics of inactivation can plausibly be explained by a reasonable mechanism for denaturation. Already the general

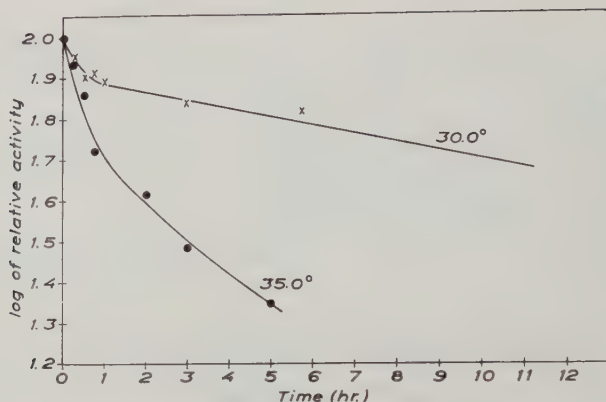


Fig. 3. The logarithm of the relative activity of carnosinase as a function of time at 30° and 35° after the addition of EDTA-Na.

appearance of the kinetic curves of the irreversible inactivation in Fig. 1 indicates that the process can scarcely be characterized as a simple first-order reaction, although the majority of denaturation processes studied follows the first-order kinetics in respect to time [9]. The semilogarithmic plot in Fig. 3 clearly reveals that the experimental points do not lie on a straight line and that the course of reaction can be best described as composed of two linear plots: one predominating in the beginning, the second determining the latter part of the curve. The simplest explanation for this kind of curve would naturally be the assumption that the enzyme preparation was inhomogeneous and that the activity was due to two enzymes (E and E') with different rates of inactivation. In this case the break in the curve of denaturation, which at 11.0° (Fig. 2) takes place very distinctly after the inactivation of 35–40 % of the enzyme, should not be found after 15–20 % of inactivation in the presence of Na^+ ions. That means, more explicitly expressed, that if we combine the equations representing the inactivation of both enzymes

$$E = E_0 \exp(-\lambda_1 t) \quad (1)$$

$$E' = E'_0 \exp(-\lambda_2 t) \quad (2)$$

to represent the total apparent inactivation [10]

$$E + E' = E_0 \exp(-\lambda_1 t) + E'_0 \exp(-\lambda_2 t), \quad (3)$$

then the constants E_0 and E'_0 represent the initial concentrations of both enzymes and should be independent of temperature. Clearly, this is not so in the case of the inactivation of carnosinase at 30° and 35° as represented by the semilogarithmic plot in Fig. 3. The kinetic evidence together with the physical criteria for protein homogeneity [3] definitely exclude the possibility of the existence of several enzymes.

The same type of kinetic curves as those which carnosinase shows has previously been described by Wright and Shomaker [11], who studied diphtheria antitoxin, and by Chase [12] in his studies of luciferase. They explain the form of the denaturation curves by assuming the following scheme for denaturation



where N stands for the native protein, P for the protected form in equilibrium with N , and I for the irreversibly denaturated protein.

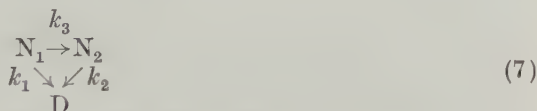
By solving the system of differential equations corresponding to the scheme above, Wright and Shomaker showed that the time course of irreversible denaturation was represented by

$$N = N'_1 \exp(-\lambda_1 t) + N'_2 \exp(-\lambda_2 t) \quad (5)$$

N'_1 and N'_2 being constants dependent on temperature and the initial concentration of the protein. The experimental points of carnosinase inactivation fit adequately an equation of this type. Chase [12] could by independent experiments in the case of luciferase demonstrate the existence of the reversible step $N \rightleftharpoons P$. In the case of carnosinase the nonexistence of a lag period means that either there is no reversible step of the luciferase type or both forms N and P are active differing only in stability. Thus in order to accept Wright and Shomakers scheme for carnosinase inactivation we have to postulate the existence of an active stable form P and a reversible step $P \rightleftharpoons N$. There is no independent evidence for either of these postulates. The inactivation of carnosinase can be explained more plausible by the following scheme



where N_1 and N_2 are two different forms of the enzyme and D the irreversibly denaturated enzyme. This scheme contains no stable active form and the existence of a reversible step is not indispensable as scheme (6) can equally well be completely irreversible:



N_1 and N_2 are both assumed to be capable of being activated, the concentration of N_2 being 0 at t_0 . This triangle scheme gives the following system of differential equations

$$\left\{ \begin{array}{l} \frac{dN_1}{dt} = -(k_1 + k_3)N_1 + k_4N_2 \\ \frac{dN_2}{dt} = -(k_4 + k_2)N_2 + k_3N_1 \\ \frac{dD}{dt} = k_1N_1 + k_2N_2 \end{array} \right. \quad (8)$$

which is a case of the general first-order series and parallel reaction as described by Frost and Pearson [13]. The solution of the equations according to Frost and Pearson yields equation (9) which expresses the remaining activity as a function of time and is in form identical with equation (5)

$$N_1 + N_2 = A \exp(-\lambda_1 t) + B \exp(-\lambda_2 t) \quad (9)$$

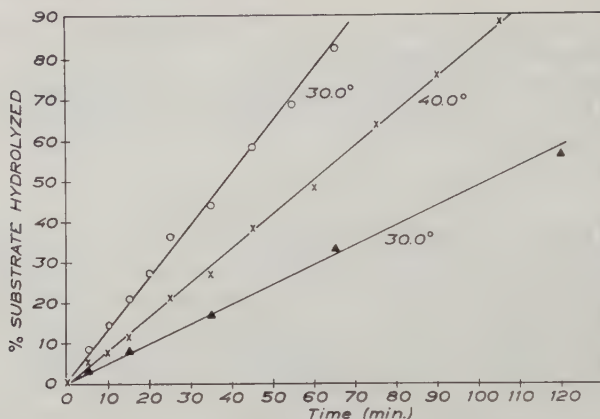
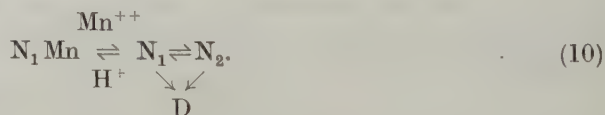


Fig. 4. The hydrolysis of carnosinase at pH 7.65 with electrodialyzed, metal-ion-free enzyme when the activating Mn^{++} ions were added at $t=0$. $\times - \times - \times$ and $O - O - O$ the enzyme was added immediately after the end of electrodialysis. $\blacktriangle - \blacktriangle - \blacktriangle$ the electrodialyzed enzyme was stored in metal-ion-free solution at 10° for 30 hr before the experiment.

A and B are functions of $k_1, k_2, k_3, k_4, \lambda_1, \lambda_2$ and of the initial concentration of the enzyme. Fig. 12 shows the experimental results at 35° fitted to an equation of this type.

The existence of two forms of the enzyme must be explained as follows; if at constant pH we replace the divalent ions by monovalent THAM, Na^+ or hydrogen ions, the enzyme conformation changes to another form N_2 , which is the more stable one in the absence of divalent ions. If this were true, we should achieve the same rearrangement just by lowering, in the presence of the metal ions, the pH enough to replace most of the divalent ions by H^+ . Fig. 6 shows the inactivation at pH 6 in the presence of 0.01 Mn^{++} . The time course of this inactivation is in accordance with scheme (6) or (7) when the interaction with Mn^{++} ions is included:



If we replace Mn^{++} with a more general Me^{++} , the scheme above represents the inactivation of carnosinase in the presence of divalent metal ions.

The studies of the specificity of metal-ion stabilization of carnosinase were not aimed at cataloguing the influence of all divalent ions but at giving an answer to definite questions in connection with studies of activation of carnosinase by divalent metal ions. The results in Fig. 5 clearly show that metal ions such as Ca^{++} and Mg^{++} do show stabilizing properties, although they do not activate the enzyme. As the interaction of Mn^{++} , Ca^{++} and Mg^{++} ions with the buffer is negligible, the free metal-ion concentration is approximately the same, and differences in inactivation curves correspond to differences in stabilizing properties according to the series $Mn^{++} > Ca^{++} > Mg^{++}$.

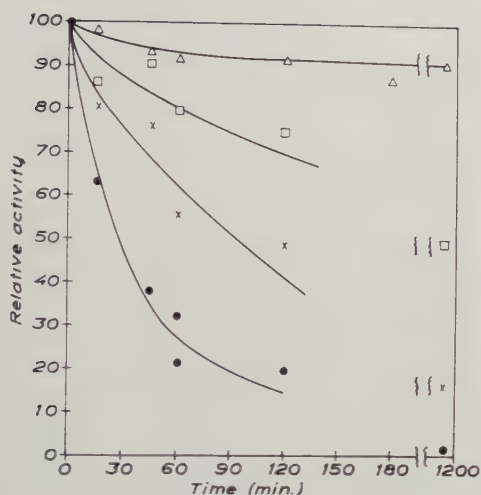


Fig. 5.

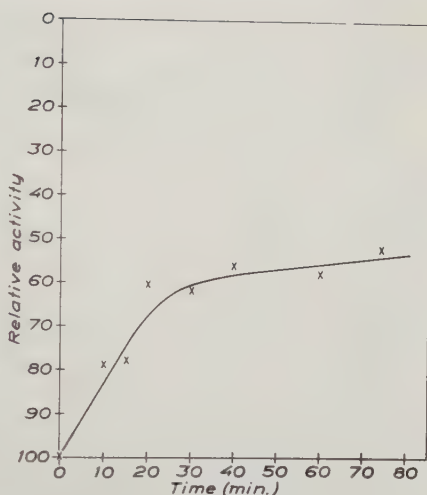
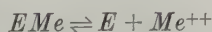


Fig. 6.

Fig. 5. The irreversible inactivation of electro dialyzed carnosinase at 40°, pH₄₀ 7.65 and in presence of $\Delta - \Delta - \Delta$ 5×10^{-3} M Mn⁺⁺, $\square - \square - \square$ 5×10^{-3} M Ca⁺⁺, $\times - \times - \times$ 5×10^{-3} M Mg⁺⁺ and in metal-ion-free solution $\bullet - \bullet - \bullet$.

Fig. 6. The irreversible inactivation of carnosinase at pH₄₀ 6.0 and at 40°, in presence of 0.01 M Mn⁺⁺.

Fig. 8, showing the stabilizing properties of Cd⁺⁺ and Zn⁺⁺ ions, indicates that the metal activating the enzyme does not necessarily stabilize it. This is especially conspicuous for Cd⁺⁺ ions, which at the concentration used give maximal activation of the enzyme [4]. Normally this would be sufficient to prove that the activating and stabilizing functions of divalent metal ions do not coincide. Unfortunately the results are complicated by the appearance of precipitates of protein after the addition of Zn⁺⁺ or Cd⁺⁺. The most plausible explanation is, of course, that Zn⁺⁺ and Cd⁺⁺ precipitate the denatured form of the enzyme, which already in a metal-ion free solution shows a considerable decrease in solubility. This, however, does not exclude the possibility, that although Cd⁺⁺ and Zn⁺⁺ activate the enzyme and simultaneously even stabilize it, the binding of Cd⁺⁺ and Zn⁺⁺ to other sites in protein enhances the inactivation of the enzyme. Such increase in the rate of denaturation by Cd⁺⁺ and Zn⁺⁺ has been observed with pepsin [14]. In order to compensate for this uncertainty in the interpretation of the different effects, the stabilization by Mn⁺⁺ and Ca⁺⁺ has been studied more thoroughly. By comparing two ions, one activating the enzyme and the others not, but both stabilizing the enzyme, a more definite conclusion may be drawn about the difference in activation and stabilization sites. The influence of the concentration of these ions on the stability is compared in Fig. 10. A rough estimate for the magnitude of the constant K_{eq} in the assumed equilibrium



gives the values of 2×10^{-5} M for Mn⁺⁺ and 1×10^{-4} M for Ca⁺⁺. This means that the stabilization by Mn⁺⁺ takes place approximately in the same range of concen-

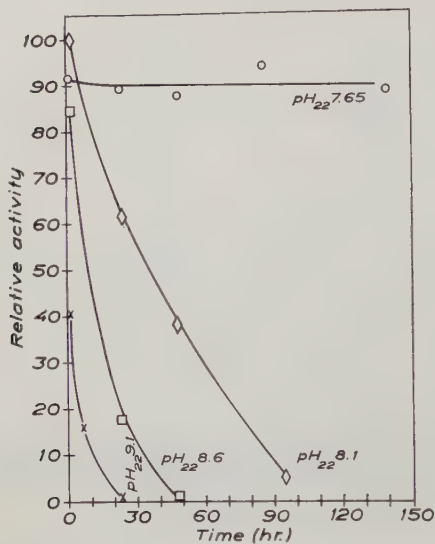


Fig. 7.

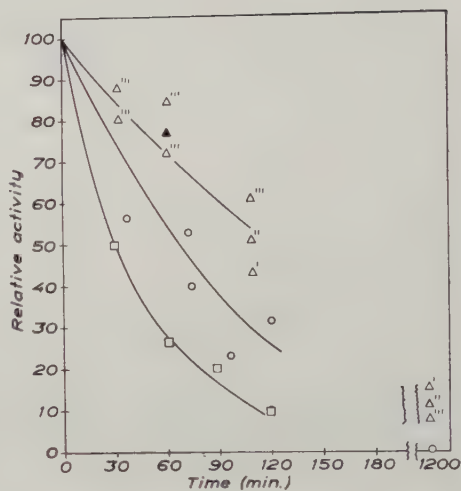


Fig. 8.

Fig. 7. The irreversible inactivation of carnosinase at 22° in presence of 0.01 M Mn²⁺.

Fig. 8. The irreversible inactivation of electro dialyzed carnosinase at 40°, pH₄₀ 7.0 in presence of $\bigcirc - \bigcirc - \bigcirc$ 1×10^{-3} M Zn²⁺, $\triangle'$ 3.3×10^{-4} M Cd²⁺, $\triangle''$ 6.6×10^{-4} M Cd²⁺, $\triangle'''$ 1.3×10^{-3} M Cd²⁺, $\blacktriangle$ 1×10^{-4} M Cd²⁺ and in metal-ion-free solution $\square - \square - \square$.

tration as activation. These values for constants are very approximate, as it is by no means self-evident that the measured parameter (residual activity after 1 hour), is strictly proportional to the amount of enzyme bound to the metal ions. As the difference between the estimates of the two K_{eq} is not too large, it ought to be possible to study Ca²⁺ inhibition of Mn²⁺ activation and thereby determine if the site for activation is identical with the site for stabilization or if we have two independent sites as proposed after the study of the activation [4]. The inhibition studies are presented in Fig. 11. The plot fitting the results best at 3 different Mn²⁺ concentrations is a noncompetitive

$$\frac{v_0}{v} = 1 + \frac{[Ca^{2+}]}{K_i} \quad (11)$$

with $K_i = 0.23$. The dispersion of the experimental points does not exclude the possibility that the inhibition is partially competitive. If it is assumed to be entirely competitive according to

$$\frac{v_0}{v} = 1 + \frac{K_a [Ca^{2+}]}{K_i (K_a + [Mn^{2+}])} \quad (12)$$

where K_i is approximately 1×10^{-2} , when K_a is 4×10^{-5} [4] and $[Mn^{2+}]_{tot} = 5 \times 10^{-4}$ M, this means that, whereas stabilization takes place at $[Ca^{2+}] = 10^{-4}$ M, inhibition takes place at $[Ca^{2+}] = 10^{-2}$ M. This, together with evidence from activation studies [4] and the results from experiments with Cd²⁺ and Zn²⁺ stabilization, confirms the

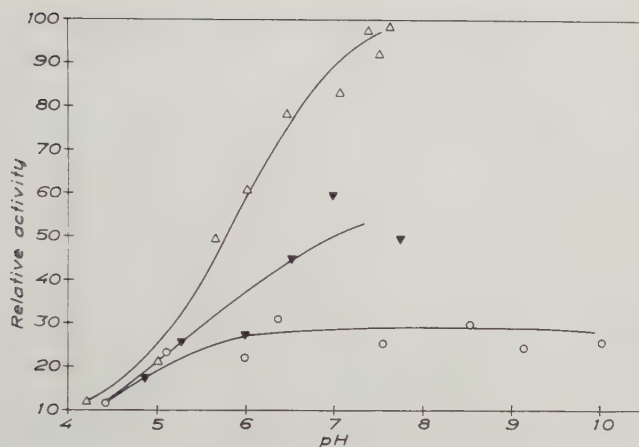


Fig. 9. The irreversible inactivation of carnosinase at 40° as a function of pH in presence of $\triangle-\triangle-\triangle$; $5 \times 10^{-3} M$ Mn^{++} , $\blacktriangledown-\blacktriangledown-\blacktriangledown$ $1 \times 10^{-3} M$ Cd^{++} , and in metal-ion-free solution $\circ-\circ-\circ$.

hypothesis of two different sites of interaction for activating and stabilizing metal ions. If we now assume that stabilizing is due to a binding of metal ions to a certain site it is interesting to note that even at the highest Ca^{++} concentrations the rate of inactivation is still higher than in the presence of Mn^{++} ions. A part of this effect may of course be due to an effect from the very high Ca^{++} ion concentration. Irrespective of that, it is by no means necessary that the Ca -enzyme compound is as stable as the Mn -enzyme compound. Here the binding of Mn ions to the site of activity (provided the activation takes place primarily through a Mn -enzyme complex) probably lends extra stability to the enzyme structure.

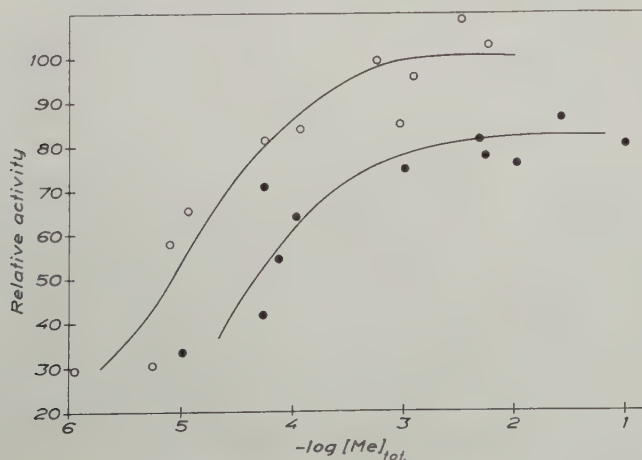


Fig. 10. The loss of carnosinase activity after 1 hr at pH_{40} 7.65 and at 40° as a function of the negative logarithm of the concentration of $\circ-\circ-\circ$ Mn^{++} and $\bullet-\bullet-\bullet$ Ca^{++} .

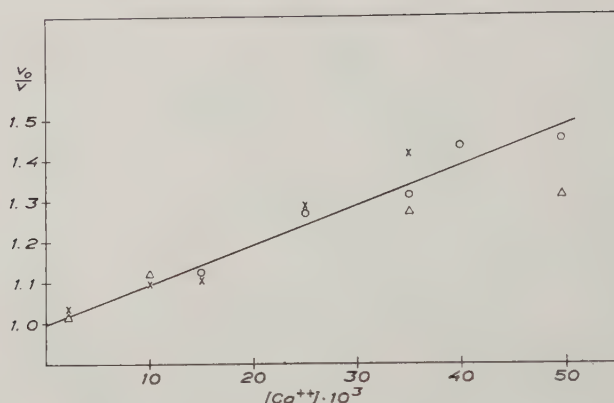


Fig. 11. The inhibition of Mn^{++} -activated carnosinase by Ca^{++} at 40° and at pH_{40} 7.65 expressed as a function of $[\text{Ca}^{++}]$ at different concentrations of Mn^{++} . $\times - \times - \times$ 3×10^{-4} M Mn^{++} , $\circ - \circ - \circ$ 6×10^{-4} M Mn^{++} , $\triangle - \triangle - \triangle$ 1×10^{-3} M Mn^{++} .

The stabilization of protein structure by divalent ions and especially by Ca^{++} and Mn^{++} has been previously reported for lysozyme [15], trypsin [16, 17], α -amylase [18] and pancreatic lipase [19]. These are certainly not the only cases of metal-ion stabilization of proteins, although few studies of protein stability in connection with the control of metal ions have been carried out up to date. Consequently no theory about the nature of this stabilization has been forwarded except for the general assumption of the existence of salt bonds in the secondary and tertiary structure of proteins [20].

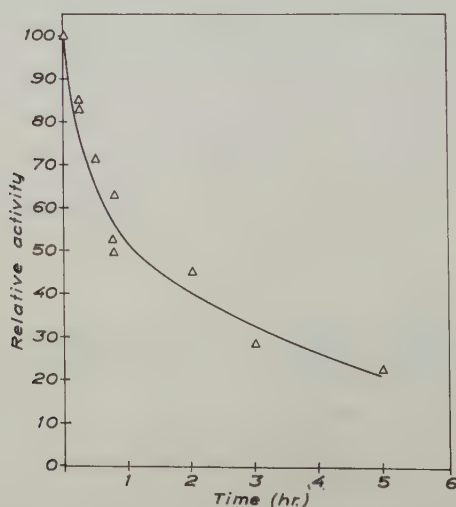


Fig. 12. Comparison of the irreversible inactivation of metal-ion-free carnosinase at pH_{35} 7.65 and at 35° with the equation

$$E = 40 \exp(-0.044 t) + 60 \exp(-0.00345 t)$$

where E is the residual activity and t the time (min).

Nearest to an explanation is the discussion by Ruegamer [21] of the Ca^{++} stabilization of coliphage. From kinetic data Ruegamer concludes that stabilization takes place by Ca^{++} stoichiometrically replacing two hydrogen ions from the surface of the coliphage since the velocity constant of denaturation is inversely proportional to Ca^{++} and proportional to $[\text{H}^+]^2$. If stabilization is effected by chelate formation on the surface of coliphage, the kinetic results are as expected. The failure of Na^+ ions to follow the same type of law is by no means unfortunate for the theory [22], but rather confirms it as chelate formation is excluded for univalent ions. The stabilization of carnosinase fits nicely to this picture. The only difference is that, whereas, according to Ruegamer, all divalent ions investigated (e.g. Mn^{++} , Mg^{++} and Zn^{++}), can replace Ca^{++} ions, there is an essential difference between Mn^{++} and Zn^{++} , for example, in the case of carnosinase. The greater divergency between the observed effect of stabilization is probably due to higher pH of the experiments with carnosinase. At pH lower than 4.6, as used for coliphage, probably only COO^- groups are available for chelate formation. At pH 7.65 a much greater number of combinations is available for this purpose.

The number of ions per molecule necessary for stabilization of the groups partaking in chelate formation cannot be determined on the basis of the experiments presented here. Further progress depends mainly on the elimination of the two chief experimental weaknesses of this work, namely the slow and insensitive method for activity determinations and the lack of a suitable physical method of following changes in protein structure independent of activity measurements.

SUMMARY

The kinetics of the irreversible inactivation of carnosinase in metal-ion free solutions has been studied. The results are best explained by assuming the existence of an equilibrium between different forms of the enzyme, the rate of inactivation being different for each form. The nature of the process of inactivation must be described as thermal denaturation of the protein molecules without any extensive hydrolysis of the peptide bonds of the protein. All the divalent ions investigated influence the rate of inactivation. A comparison of the effect of some divalent ions results in a series arranged according to the stabilizing capacity of the ions: $\text{Mn}^{++} > \text{Ca}^{++} > \text{Mg}^{++}$ ($= \text{Cd}^{++} > \text{Zn}^{++}$). A direct comparison of the stabilizing capacity of Cd^{++} and Zn^{++} ions is hindered by complications arising from the appearance of protein precipitates in the presence of these ions.

Studies of metal-ion stabilization as a function of the concentration of the stabilizing ion showed the concentration range where the stabilization took place. The results were then compared with studies of Ca^{++} inhibition of the Mn^{++} -activated enzyme. It could be shown that inhibition by Ca^{++} ions took place at much higher concentrations than stabilization. The simplest explanation of these results is the existence of two different sites, one for activation, the other for stabilization. The apparent specificity of activation is thus a combination of two different functions. The nature of the interaction of stabilizing metal ions with the protein molecule is supposed to be chelate formation. No evidence was found for the existence of a lag period in the metal-ion activation of the enzyme.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Professor Arne Tiselius for his encouraging interest and to make grateful acknowledgement to Mr. Stig Bergvall for invaluable technical assistance. This investigation is part of a research program on the structure and activity of metal enzymes financially supported by the Swedish Natural Science Research Council and the National Institutes of Health, U.S. Public Health Service (RG-6542).

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Tryckt den 18 november 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB

Studies on carnosinase

IV. The interaction of the activating metal ions with the substrate

By ANDREAS ROSENBERG

With 5 figures in the text

Introduction

The formation constants of carnosine (β -alanyl-L-histidine) complexes with Cd^{++} and Zn^{++} ions have recently been determined by Martin and Edsall [1]. The authors used the potentiometric titration method and after assuming that the initial interaction takes place at the pyridine nitrogen of the imidazole, calculated the successive formation constants. In the pH region relevant to studies of carnosinase action, no chelation was assumed to take place. Zn^{++} -carnosine interaction was characterized by association constants which were greater than expected on statistical grounds. This abnormal behaviour of the Zn^{++} complexes was of the same magnitude as observed for Zn^{++} -imidazole complexes.

The aim of this investigation is to supplement the above-mentioned results with additional data necessary for the discussion of the metal-ion activation of carnosinase [2]. The interaction of carnosine with Mn^{++} ions has been studied by electron spin resonance (ESR). The temperature dependence of Cd^{++} -carnosine interaction was determined by potentiometric titrations and the metal-indicator method of Raaflaub has been used to check the results from the potentiometric titrations.

Methods and materials

The concentration of free Mn^{++} ions in solutions of MnCl_2 and carnosine was determined by measurements of electron spin resonance (ESR). The technique of the ESR measurements of Mn^{++} concentration was the same as described by Malmström, Vänngård and Larsson [3]. The measurements were performed with a Varian V-4500 spectrometer. The samples were kept in quartz tubes of 1.3 mm bore.

The potentiometric titrations were carried out with a di-functional titrator from International Instrument Co. The titrator was equipped with a Leeds and Northrup pH-meter, model 7664. The glass electrode and the calomel electrode were from Beckman Inc. The response of the glass electrode was calibrated by comparison with 0.05 *M*. KH-phthalate buffer. The pH of the phthalate buffer was assumed to be 4.01 at 25° and 4.03 at 40° [4]. Before measurements at 40° the electrodes were kept in phthalate buffer at 40° until the reading on the pH meter stabilized. Solutions

of 0.03 *M* carnosine nitrate and 0.015 *M* CdSO₄ were titrated with 1 *N* NaOH added from the Agla syringe of the titrator. In addition to N₂ bubbled through the solutions, magnetic agitation was used to create an effective stirring. The titration vessel was equipped with a water jacket connected to a thermostatically controlled water-bath.

As the aim of these titrations was not a precise determination of complex constants but a comparison of the complex formation at different temperatures and at conditions resembling the activity measurements, the ionic strength was not brought to $\mu = 0.16$ as was the case for measurements of Mn⁺⁺ binding and the Raaflaub determinations.

The technique of metal-indicator analysis has been described by Raaflaub [5]. The only difference in this case was that instead of determining the metal-murexide association constant prior to calculation of the formation constants, a calibration curve of free-metal-ion concentration versus optical density was used to estimate the free-metal-ion concentration. The pH of the calibration solutions and the solutions investigated was kept constant by addition of THAM¹-acetate or THAM-maleate buffers. The influence of buffer on the free metal ion concentration was determined separately. The estimation of buffer-metal interaction is the main source of error for the determination of Cd⁺⁺ and Zn⁺⁺ concentrations, since, owing to difficulties in keeping the pH constant, the interaction with buffer can be studied only over a relatively narrow range of concentrations. The indicator concentration was 1.5×10^{-5} *M* for determinations of Cd⁺⁺ at pH₂₅ 7.65² and 4.5×10^{-5} *M* for determinations of Zn⁺⁺ at pH₂₅ 6.86. The ionic strength was kept at 0.16 in all solutions by addition of 0.16 *M* KNO₃. The optical density corresponding to Cd⁺⁺-murexide was measured at 470 m μ and Zn⁺⁺-murexide at 454 m μ .

Chromatographically pure L-carnosine from Th. Schuchardt was used either directly or as nitrate crystallized from water and ethanol. Analytical grade salts were used to prepare buffers and metal-ion solutions. The buffers and carnosine solutions were purified by extraction with dithizone [2]. Murexide was used in form of ammonium purpureate from B.D.H.

Results

The measurements of Mn⁺⁺-carnosine interaction were performed with solutions of constant total Mn⁺⁺ content and varying concentrations of carnosine. Carnosine solutions were adjusted to pH₃₆ 7.65 and mixed with the metal ion solution. The pH of the resulting solution was checked with a pH-meter. The pH values for different concentrations of carnosine varied by ± 0.02 pH units. All the solutions used contained 0.16 *M* KNO₃. Solutions of known content of MnCl₂ in 0.16 *M* KNO₃ were used for calibrating purposes. The total concentration of Mn⁺⁺ in the measurements in the presence of carnosine was 1×10^{-3} *M*. The temperature of the cavity was $36 \pm 2^\circ$. The temperature variations introduce an extra uncertainty of approximately 0.03 pH units in the pH determinations. For the interpretation of the results it was assumed that the complex-forming species of carnosine is the dipolar ion with no charge at the pyridine N of the imidazol ring. This means that at pH 7.65 the interaction of Mn⁺⁺ ions with the amino group was neglected.

The best fit between the observations and a calculated curve was obtained when it was assumed that the complex formation was dominated by the first step:

¹ THAM = Tris(hydroxymethyl)aminomethane.

² The subscript 25 indicates the temperature at which the pH of the solution was determined.

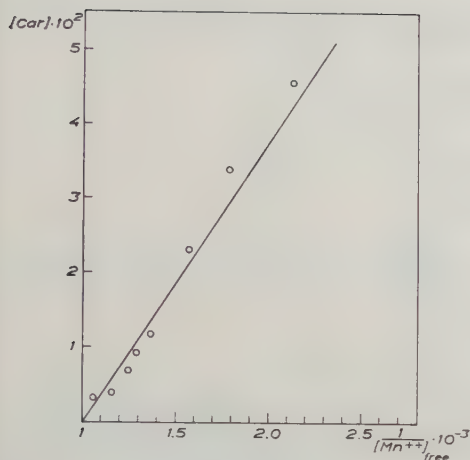


Fig. 1.

Fig. 1. The inverse concentration of free Mn^{++} ions as a function of free carnosine concentration (equation (3)). The straight line represents a calculated curve based on $k_1 = 27$. The total Mn concentration was $1 \times 10^{-3} M$, the temperature $36 \pm 2^\circ$ and the ionic strength 0.16.

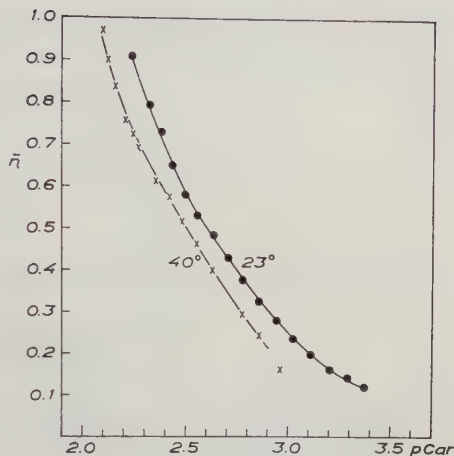


Fig. 2.

Fig. 2. The formation curves of carnosine-cadmium complexes at 23° and 40° , calculated from the titration results from Table II according to equations (4)–(6).

$$k_1 = \frac{[\text{Mn}^{++} \text{ Car}]}{[\text{Car}] [\text{Mn}^{++}]} \quad (1)$$

Car notifies the concentration of the free ligand. The observed concentration of the free Mn^{++} ions can then be represented by

$$[\text{Mn}^{++}] = \frac{[\text{Mn}_{\text{tot}}]}{1 + k_1 [\text{Car}]} \quad (2)$$

The results can advantageously be plotted in the form of the reciprocal of equation (2):

$$\frac{1}{[\text{Mn}^{++}]} = \frac{1}{[\text{Mn}_{\text{tot}}]} + \frac{k_1}{[\text{Mn}_{\text{tot}}]} [\text{Car}]. \quad (3)$$

The results are shown in Fig. 1. The straight line corresponds to a k_1 value of 27. The pK_a of carnosine imidazol at 36° , used to calculate the free ligand concentrations, was interpolated from the values at 25° and 40° . The amount of carnosine bound to Mn^{++} has been omitted, since calculations showed that it was less than the error in measurements.

The value for $\log k_1$ at 36° , 1.43, is reasonable when compared to $\log k_1 = 1.75$ for histidine- Mn^{++} [6].

The ionization constants for the imidazole group of carnosine were determined separately by potentiometric titrations. The values obtained are compared in Table I with the values available in the literature.

Table 1. Apparent dissociation constants, pK_a , at various temperatures and ionic strength for the conjugate acid of the imidazole group of L-carnosine.

Ionic strength	Temperature, °C			
	23	25	30	40
0.075	6.81		6.69	6.56
0.075	6.78 ^a			
0.16		6.86 ^b		6.61

^a Deutsch and Eggelton [7].^b Martin and Edsall [1].*Table 2.* Titration of mixtures of L-carnosine nitrate and cadmium sulfate.

Results at 23°				Results at 40°			
Total molar concentration of				Total molar concentration of			
L-carnosine nitrate	CdSO ₄	NaOH	pH	L-carnosine nitrate	CdSO ₄	NaOH	pH
0.02921	0.01496	0.002246	5.00	0.03003	0.01496	0.002672	5.01
0.02920	0.01496	0.002678	5.10	0.02999	0.01494	0.004043	5.20
0.02919	0.01495	0.003129	5.20	0.02997	0.01493	0.004955	5.30
0.02917	0.01494	0.003758	5.30	0.02993	0.01491	0.006043	5.40
0.02915	0.01493	0.004443	5.40	0.02990	0.01489	0.007126	5.50
0.02912	0.01492	0.005304	5.50	0.02986	0.01487	0.008348	5.60
0.02909	0.01491	0.006241	5.60	0.02982	0.01485	0.009703	5.70
0.02906	0.01489	0.007235	5.70	0.02979	0.01483	0.01097	5.80
0.02903	0.01487	0.008401	5.80	0.02974	0.01481	0.01244	5.90
0.02899	0.01485	0.009778	5.90	0.02970	0.01479	0.01355	6.00
0.02896	0.01484	0.01074	6.00	0.02964	0.01476	0.01567	6.15
0.02893	0.01482	0.01194	6.10	0.02962	0.01475	0.01646	6.20
0.02888	0.01480	0.01336	6.20	0.02957	0.01473	0.01799	6.30
0.02884	0.01477	0.01505	6.30	0.02953	0.01471	0.01943	6.40
0.02879	0.01475	0.01639	6.40	0.02948	0.01468	0.02086	6.50
0.02872	0.01471	0.01921	6.60	0.02944	0.01466	0.02223	6.60
0.02864	0.01467	0.02164	6.80	0.02941	0.01464	0.02360	6.70

Differences between the values determined in the present work and that of Deutsch and Eggelton are partly explained by a difference in the pH scale, which, for Deutsch and Eggelton, is based on the value 3.98 for 0.05 *M* KH-phthalate buffer.

The results of the potentiometric titrations are presented in Table 2 and then plotted in form of formation curves, according to Bjerrum [8], Fig. 2. The formation constants are calculated on the assumption that the complex formation initially takes place at an imidazole nitrogen and that the interaction with the NH₂ group can be neglected. The slight upward curvature of the formation curves in Fig. 2 is probably due to the increasing influence of the NH₂ group.

The $\bar{n}$ and log Car (the logarithm of the free carnosine concentration) were calculated from the titration data according to the equations formulated by Albert [9]:

$$\log [\text{Car}] = \text{pH} - \text{pK}_a + \log ([\text{Car}_{\text{tot}}] - [\text{NaOH}]) \quad (4)$$

$$\bar{n} = \frac{[\text{Car}_{\text{tot}}] - \alpha [\text{Car}]}{[\text{Cd}_0]} \quad (5)$$

$$\alpha = \frac{[\text{H}^+]}{\text{K}_a} + 1. \quad (6)$$

Cd_0 represents the total metal ion concentration in the solution. $\log k_1$ and $\log k_2$ were then calculated from the equation

$$\bar{n} = \frac{k_1 [\text{Car}] + 2 k_1 k_2 [\text{Car}]^2}{1 + k_1 [\text{Car}] + k_1 k_2 [\text{Car}]^2}. \quad (7)$$

In view of the relative nature of the measurements, the calculations were simplified by assuming that the spreading factor for the successive formation constants was the same as determined by Martin and Edsall [1]. This simplification holds rather well for $\bar{n} = 0.2$ to 0.5 , in which range the $\log k_1$ changes no more than ± 0.03 . The association constants are presented in Table 3.

The necessity of determining the interaction of carnosine with Cd^{++} and Zn^{++} ions by other methods than potentiometric titrations arises from the discrepancy between the results from the determinations of free-metal-ion concentrations in reaction mixtures [2] and the concentrations of free metal ions calculated from the formation constants.

Calculations with the k values of Martin and Edsall show that Zn^{++} should be bound more effectively to carnosine than Cd^{++} . The free-metal-ion concentrations determined according to Raaflaub seem to indicate that Cd^{++} is bound more strongly than Zn^{++} . In order to clarify this question, the binding of Zn^{++} and Cd^{++} has been determined at constant pH as a function of the concentration of free carnosine. The metal-indicator method of Raaflaub described in the section on methods was used to determine the Cd^{++} -carnosine interaction at a constant total Cd^{++} concentration and in the presence of varying amounts of carnosine. Both the metal ion solution used for calibration and the mixtures investigated contained $1.4 \times 10^{-2} M$ THAM in the form of THAM-acetate buffer of $\text{pH}_{25} 7.65$. The binding constant of THAM- Cd^{++} was determined separately. Due to the weak binding, only k_1 could be estimated with reasonable precision. The $\text{p}k_1$ used to correct the apparent calibration curves was 1.8. The pK_a of THAM in $0.16 M \text{KNO}_3$ was determined by potentiometric titration and was found to be 8.32. Fig. 3 shows the concentration of free Cd^{++} ions

Table 3. The formation constants for L-carnosine with Cd^{++} at different temperatures and 0.75 ionic strength.

	Temperature, °C	
	23	40
$\log k_1$	2.45	2.35
$\log k_2$	1.70	1.50

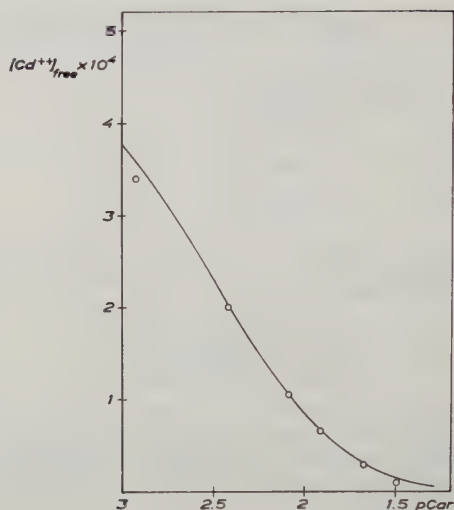


Fig. 3. The concentration of free Cd^{++} ions at pH_{25} 7.65 as a function of free carnosine concentration, determined by the metal-indicator method. The total concentration of Cd^{++} was $5 \times 10^{-4} M$. The solid line represents the theoretical curve based on k_1 and k_2 values of Martin and Edsall.

as a function of the free ligand concentration. The following equations express the relations between the formation constants and the free-metal-ion concentration:

$$[\text{Cd}_{\text{tot}}] = [\text{Cd}^{++}] (1 + k_1 [\text{Car}] + k_1 k_2 [\text{Car}]^2),$$

$$[\text{Car}_{\text{tot}}] = [\text{Car}] \left(1 + \frac{[\text{H}^+]}{K_a} + k_1 [\text{Cd}^{++}] + 2 k_1 k_2 [\text{Cd}^{++}] [\text{Car}] \right). \quad (8)$$

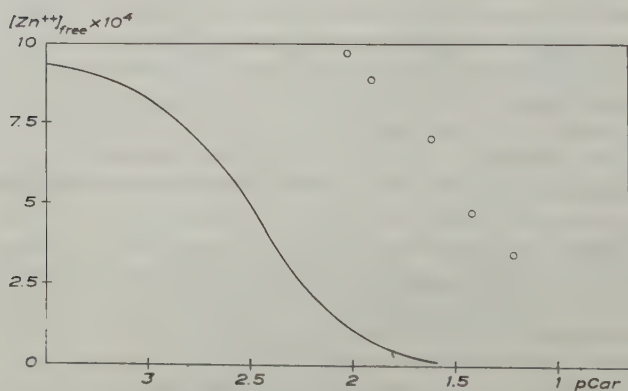


Fig. 4. The concentration of free Zn^{++} ions as a function of free carnosine concentration at pH_{25} 6.86, determined by the metal-indicator method. The total concentration of Zn^{++} was $1 \times 10^{-3} M$. The solid line represents the theoretical curve calculated from the k values of Martin and Edsall (Table 4).

Table 4. The successive formation constants for L-carnosine with Zn^{++} at 25° and an ionic strength of 0.16.

	Potentiometric titration	Metal indicator
k_1	2.30	~ 1.40
k_2	2.10	
k_3	2.00	
k_4	1.70	

The theoretical curve was calculated with $\log k_1 = 2.50$ and $\log k_2 = 1.75$. In calculations of free carnosine the carnosine bound to Cd^{++} has been neglected.

A similar determination was carried out with Zn^{++} ions. The pH in this case was 6.86 in order to avoid the formation of hydroxy compounds. The ionic strength was 0.16. The total Zn^{++} concentration in all the solutions measured was $1 \times 10^{-3} M$. The buffer used in measurements of complex formation and in calibration was THAM-maleate. The presence of buffer did not influence the measured Zn^{++} concentrations significantly and has been neglected. The measurements at the highest carnosine concentration are rather uncertain due to background absorption from the carnosine solution. The results presented in Fig. 4 differ considerably from the curve calculated with the 4 successive formation constants determined by Martin and Edsall [1]. There is no evidence for the abnormally high binding in the second, third and fourth successive complexes. The formation constants calculated from the experimental curve are compared in Table 4 with the k values from the literature.

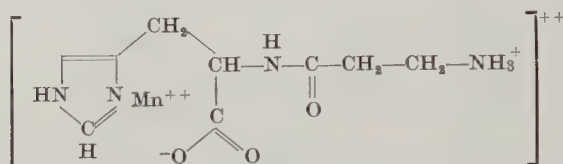
This discrepancy must either be due to the failure of the Raaflaub method or an error in the potentiometric titrations due to extensive formation of hydroxy compounds of Zn^{++} . An inspection of the absorption curves of Zn^{++} and murexide in the presence of carnosine reveals no extra absorption due to possible impurities. The apparent high concentrations of free Zn^{++} ions must be explained by assuming the formation of mixed complexes between the first carnosine- Zn^{++} complexes and murexide. However, in face of the proposed structure for the murexide metal compounds [10] this implies that the Zn^{++} -murexide compound should differ considerably from the complexes of Ca^{++} and Cd^{++} . A reasonable explanation is the assumption that the inherent errors in both methods are in the opposite directions. This is contradicted by the excellent agreement of the results for the Cd^{++} -carnosine interaction. Obviously, a detailed study of Zn^{++} -murexide interaction is necessary before any further discussion of this question.

Discussion

The binding of Mn^{++} to carnosine is characterized by a very low association constant. Since the activation of carnosinase takes place at rather low metal ion concentration ($1 \times 10^{-4} - 1 \times 10^{-5} M$), it would, at the first moment, appear as if the magnitude of $\log k_1$ directly excludes the possibility of the formation of a metallo-substrate. Calculations however show that, due to the high concentration of the substrate, the absolute amount of Mn^{++} -carnosine is of the same magnitude as the free-

metal-ion concentration. Thus the low binding capacity does not exclude the possibility of carnosine-Mn⁺⁺ acting as a metallo-substrate.

The results, on the other hand, allow us to speculate about the structure of the complex of carnosine with Mn^{++} . Since initial binding takes place at the imidazole ring and the association with the amino group can be neglected in the pH region of the enzyme activation, the structure can be represented by



This means that the possible metallo-substrate has 3 charged groups around the asymmetric α -carbon of histidine. Likewise, in case the metal ion is first bound to the enzyme, the substrate has two charged groups available for interaction with the active site. The question in which direction these groups are orientated cannot be answered from the present data. The direction of the negative carboxylate group towards the Mn^{++} ion, indicated in the figure above, is just a reasonable possibility. The fact that the groups available for interaction with the enzyme are charged ought to have a considerable influence on the thermodynamic parameters of the enzymic reaction. Thus a study of the dependence of the activation on temperature, ionic strength and dielectric constant may give more evidence about the nature of the groups involved in the enzyme action. The temperature dependence of Cd^{++} -carnosine interaction was determined in order to compare the results with the observed temperature dependence of Cd^{++} activation of carnosinase [2]. If we assume that the metal functions by formation of a metallo-substrate, then the temperature dependence of the activity measurements should be comparable to the temperature dependence of the carnosine- Cd^{++} association. The formation curves in Fig. 2 at 23° and 40° show that the change in the first association constants is 0.09 units. The k values decrease with the rise of temperature, as expected for simple stepwise complex formation. These results cannot, however, be compared with the activity measurements since the measured activity is not influenced only by the association constant but rather by the actual concentration of the complex; besides the association constant, this is also dependent on the ionisation constant of carnosine. If we calculate the actual concentration of the first Cd^{++} -carnosine complex at the two temperatures (Fig. 5) it is obvious that the change in the association constants dominates the change of the complex concentration. Thus the change of the concentration of the Cd^{++} carnosine with temperature is in the opposite direction to the change in activity when the changes are compared at the same free Cd^{++} ion concentration. Thus the results from measurements of Cd^{++} carnosine association do not give support to the metallo-substrate hypothesis. Naturally they do not exclude the possibility that the main part of the temperature dependence is due to very large changes in the binding of the metallo-substrate to the enzyme.

It is of interest to note that, whereas the first step of association of Mn^{++} and Cd^{++} with carnosine is of a similar nature, the interaction of glycylhistidine with Cd^{++} is considerably influenced by the NH_2 group [1]. This change in the complex formation of Cd^{++} with glycyl-histidine may explain the reversed effectivity of Cd^{++} and Mn^{++} as activators when glycyl-histidine is used as a substrate. Likewise the

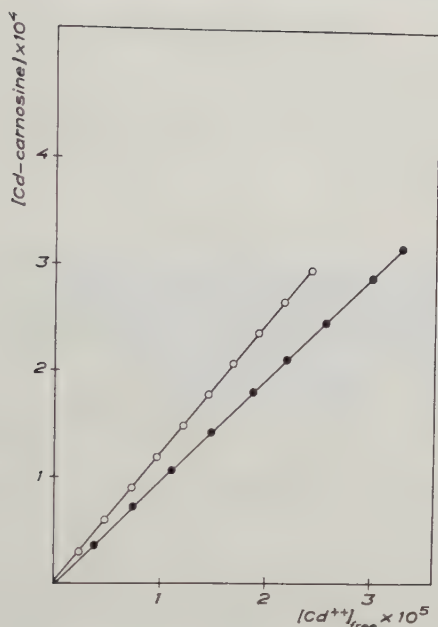


Fig. 5. The concentration of the first complex of Cd^{++} with carnosine as a function of the free metal-ion concentration when the total concentration of carnosine is $5 \times 10^{-2} M$. $\circ - \circ - \circ$ carnosine- Cd^{++} at 23° . $\bullet - \bullet - \bullet$ carnosine- Cd^{++} at 40° .

abnormal association constants of Zn^{++} -carnosine may explain the ineffectivity of Zn^{++} as an activator of carnosinase. Unfortunately the discrepancy between the titration data of Zn^{++} interaction and the results from Raaflaub determinations does not permit any speculation on the specific behaviour of Zn^{++} until the source of error is determined. This discussion of the metal-ion specificity of carnosinase and the difference in complex formation with carnosine naturally cannot indicate the path of the enzymic reaction. It only emphasizes the correlation between the interaction of metal ions with the substrate and the activating properties of the same ions.

SUMMARY

1. The formation constant for the Mn^{++} -carnosine complex was determined by measuring the electron spin resonance of the free Mn^{++} ions. $\log k_1$ was found to be 1.43.
2. The temperature dependence of the formation constants of Cd^{++} -carnosine complexes was determined by potentiometric titrations at 23° and 40° . The k value decreased with the increase of temperature as expected for simple complex formation.
3. The interaction of Cd^{++} and Zn^{++} ions with carnosine was determined by the metal-indicator method of Raaflaub. The results with Cd^{++} ions were in excellent agreement with the results from potentiometric studies. The result with Zn^{++} ions differed considerably from the theoretical curves calculated from the titration data.
4. The results were discussed with reference to the metal-ion activation of carnosinase.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Prof. Arne Tiselius for his encouraging interest and to acknowledge Mr. Stig Bergvall's invaluable technical assistance. This investigation is part of a research programme on the structure and activity of metal enzymes financially supported by the Swedish Natural Science Research Council and the National Institutes of Health, U.S. Public Health Service (RG-6542).

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Tryckt den 18 november 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB

Inhibition of uracil-utilizing enzymes by 5-fluorouracil and 5-fluorouracil nucleosides

By OLA SKÖLD

With 4 figures in the text

The pyrimidine analogue 5-fluorouracil, originally introduced as an antimetabolite by Heidelberger *et al.* [1], has been shown to have pronounced growth-inhibiting properties. Comprehensive metabolic studies with Ehrlich ascites tumor [2–5] have established two mechanisms for its growth-inhibiting effect: (a) interference with DNA¹ synthesis by the inhibition of thymidylate synthetase thus preventing synthesis of DNA-thymine in growing cells, and (b) formation of a “fraudulent” RNA containing 5-fluorouracil *in lieu* of uracil.

A third inhibition mechanism has been suggested from both *in vivo* and *in vitro* experiments which showed that the incorporation of uracil into RNA of the Ehrlich ascites tumor was inhibited by 5-fluorouracil [4, 5]. Such an incorporation of uracil into RNA has earlier been shown to proceed *via* uridine and UMP and involves the combined action of uridine phosphorylase and kinase [6, 7].

In a further study the Ehrlich ascites tumor was shown [8] to contain relatively high activities of deoxyuridine phosphorylase and deoxyuridine kinase, indicating a similar pathway to DNA *via* deoxyuridine and dUMP.

In a preliminary report [9] a pronounced inhibition of uridine phosphorylase by 5-fluorouracil was observed with a crude acetone powder extract from the tumor, suggesting that the earlier mentioned decreased incorporation of uracil into RNA in the presence of 5-fluorouracil was due to such an effect.

The aim of the present investigation was to study in more detail the inhibition by 5-fluorouracil and its nucleosides of some of the enzymes of this alternative pathway for pyrimidine synthesis. Inhibition studies were thus performed with purified preparations of uridine phosphorylase, deoxyuridine phosphorylase and uridine kinase.

¹ The following abbreviations are used in this paper: RNA = ribosenucleic acid, DNA = deoxyribosenucleic acid, UMP = uridine-5'-monophosphate, dUMP = deoxyuridine-5'-monophosphate, ATP = adenosine-5'-triphosphate, PCA = perchloric acid, tris = tris(hydroxymethyl)aminomethane chloride buffer, K_m = dissociation constant of enzyme-substrate complex, K_i = dissociation constant of enzyme-inhibitor complex, V_{max} = maximum reaction velocity at saturation of enzyme.

Experimental

Materials

5-Fluorouracil, 5-fluoroorotic acid, 5-fluorouridine and 5-fluorodeoxyuridine were obtained through the courtesy of the Hoffman-La Roche Co. Uracil-2-¹⁴C and non-labelled nucleosides were purchased from Schwarz Laboratories Inc. Uridine-2-¹⁴C and deoxyuridine-2-¹⁴C were prepared enzymically from uracil-2-¹⁴C and non-labelled nucleosides as described earlier [8]. Ribose-1-phosphate was synthesized enzymically as described by Plesner and Klenow [11].

The uridine phosphorylase preparation was the ammonium sulfate fraction from the procedure of Pontis and Reichard [12]. This preparation shows an about 40-fold purification of both uridine phosphorylase and deoxyuridine phosphorylase activities over a crude acetone powder extract from Ehrlich ascites tumor.

Uridine kinase was purified ca. 130-fold from an acetone powder extract of Ehrlich ascites tumor according to the procedure described earlier [13].

Determination of enzyme activities

Uridine phosphorylase

The activity of this enzyme was determined both by measuring the phosphorolytic cleavage of uridine and the synthesis of uridine from uracil + ribose-1-phosphate. In the first case the procedure of Pontis and Reichard [12] was used. Ten μ moles of potassium phosphate buffer, pH 7.4, 0.05 ml of enzyme, uridine and inhibitor, in different amounts, were incubated at 37° for 15 minutes (final volume = 0.1 ml). The reaction was stopped by the addition of 0.1 ml of 0.8 *M* PCA and 4 mg of charcoal (Norite A). After centrifugation 0.1 ml of the supernatant was analyzed for ribose according to Mejbaum [14]. The observed extinction at 670 m μ , read against a blank sample (PCA added prior to incubation), was taken as an expression for enzyme activity.

When synthesis of uridine from ribose-1-phosphate + uracil was measured, the following conditions were used: 0.9–1.8 μ moles of ribose-1-phosphate, 10 μ moles of tris, pH 7.4, 0.05 ml of enzyme, uracil-2-¹⁴C (590,000 c.p.m./ μ mole) and inhibitor were incubated for 15 minutes at 37° (final volume = 0.26 ml). The reaction was stopped with 0.03 ml of 4 *M* PCA, the supernatant neutralized with KOH, and KClO₄ allowed to precipitate. The final supernatant was analyzed by paper chromatography in a borate solvent as described earlier [8]. Enzyme activity, expressed as μ moles of uridine-2-¹⁴C formed, was thus calculated from the relative amount of radioactivity in the uridine spot in each chromatographic run.

Deoxyuridine phosphorylase

The procedure of Pontis and Reichard [12] was used. Ten μ moles of potassium phosphate buffer, pH 6.5, 0.02 ml of enzyme, deoxyuridine and inhibitor were incubated at 37° for 15 minutes (final volume = 0.1 ml). The reaction was stopped by the addition of 0.1 ml of 0.8 *M* PCA and 0.1 ml of the supernatant was analyzed for deoxyribose according to the method of Waravdekar and Saslaw [15]. The extinction at 532 m μ read against a blank sample (PCA added prior to incubation) was taken as an expression for enzyme activity.

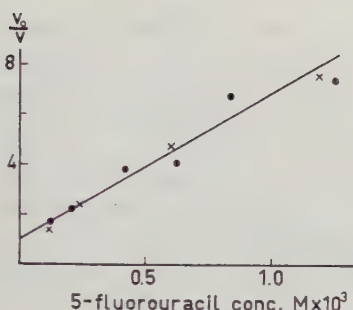


Fig. 1. Inhibition of uridine phosphorolysis by 5-fluorouracil.

A (●). Twenty μ moles of potassium phosphate buffer, pH 7.4, 0.42 μ moles (final concentration = $1.75 \times 10^{-3} M$) of uridine-2- ^{14}C (90,600 c.p.m./ μ mole), extract from 0.54 mg of acetone powder and 5-fluorouracil as indicated were incubated for 20 minutes at 37° (final volume = 0.24 ml).

B (×). Ten μ moles of potassium phosphate buffer, pH 7.4, 0.36 μ moles (final concentration = $3.6 \times 10^{-3} M$) of uridine-2- ^{14}C (81,500 c.p.m./ μ mole), 0.05 ml of purified enzyme and 5-fluorouracil as indicated, were incubated for 15 minutes at 37° (final volume = 0.1 ml).

Both experiments A and B were analyzed by paper chromatography (see Experimental).

Results

Inhibition of uridine phosphorylase

In preliminary experiments the inhibition of uridine phosphorolysis was studied in a crude acetone powder extract from Ehrlich ascites tumor, which was prepared as described earlier [6, 7]. Table I shows that 5-fluorouracil was considerably more active as an inhibitor than uracil, which is one of the products of the enzymic reaction. 5-Fluoroorotic acid showed only a negligible inhibitory effect.

Fig. 1 shows the results of an experiment in which the inhibition of uridine phosphorolysis by 5-fluorouracil was determined with both crude extract and a purified uridine phosphorylase preparation. The ratio between the velocities in the control experiments and the inhibited experiments is plotted against 5-fluorouracil concentration. Fifty per cent inhibition was obtained at a 5-fluorouracil concentration of $0.17 \times 10^{-3} M$ ($=K_i$ if non-competitive inhibition is at work) with both crude and purified enzyme.

For comparison the inhibition of purified uridine phosphorylase by uracil was investigated in a similar manner using ribose determinations as the assay. A 50% inhibition was observed at a uracil concentration of $1.0 \times 10^{-3} M$, i.e. at a sixfold higher concentration than in the 5-fluorouracil experiment.

A more detailed study on the observed inhibition by 5-fluorouracil was performed with purified uridine phosphorylase. Fig. 2 demonstrates the Lineweaver-Burk diagrams representing different concentrations of 5-fluorouracil. The straight lines obtained intersect at a common point on the abscissa, indicating inhibition of a non-competitive type. K_i -values of $0.19 \times 10^{-3} M$, $0.15 \times 10^{-3} M$ and $0.15 \times 10^{-3} M$ were calculated from curves B, C and D, respectively.

The above experiments were all performed at pH 7.4. Some experiments were also carried out at pH 6.5. The systems were identical to those described in the legend to Fig. 2 but for pH. Also at pH 6.5 a non-competitive inhibition was observed. Calculations

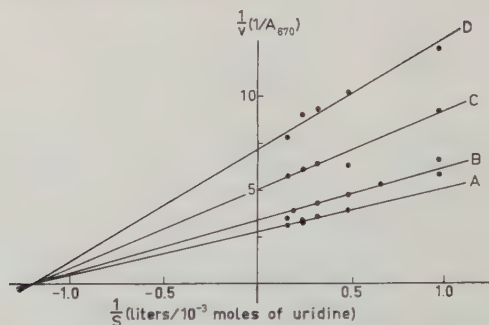


Fig. 2. Lineweaver-Burk plot of the inhibition of purified uridine phosphorylase by 5-fluorouracil. Method of assay was ribose determinations as described in Experimental. Curve A, no inhibitor; Curve B, $0.04 \times 10^{-3} M$; Curve C, $0.12 \times 10^{-3} M$ and Curve D, $0.24 \times 10^{-3} M$ of 5-fluorouracil, respectively.

lated K_i -values were $0.17 \times 10^{-3} M$, and $0.19 \times 10^{-3} M$ for 5-fluorouracil concentrations of $0.12 \times 10^{-3} M$ and $0.24 \times 10^{-3} M$, respectively.

The inhibitory effect of 5-fluorouridine on uridine phosphorolysis was studied with a system identical to that described for experiment B in Fig. 1, exchanging 5-fluorouridine for 5-fluorouracil. A 50 % inhibition was observed at a 5-fluorouridine concentration of $2.60 \times 10^{-3} M$.

The uridine phosphorylase reaction was also studied in the direction of synthesis. It was found that 5-fluorouracil inhibited the formation of uridine from uracil and ribose-1-phosphate in a non-competitive manner (Fig. 3). The calculated K_i -values from the Lineweaver-Burk plots were $0.15 \times 10^{-3} M$ and $0.07 \times 10^{-3} M$ for 5-fluorouracil concentrations of $0.046 \times 10^{-3} M$ and $0.069 \times 10^{-3} M$, respectively.

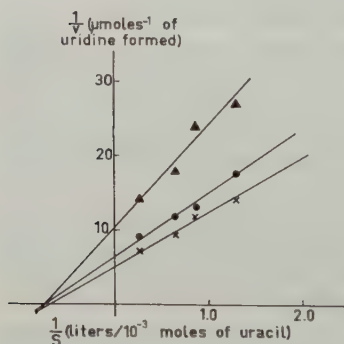


Fig. 3.

Fig. 3. Lineweaver-Burk plot of the inhibition by 5-fluorouracil of uridine synthesis from uracil-2- ^{14}C and ribose-1-phosphate (Purified uridine phosphorylase preparation). For method of assay, see the text (Experimental). $- \times - \times - \times -$ no inhibitor present, $- \bullet - \bullet - \bullet -$ 5-fluorouracil $0.046 \times 10^{-3} M$ and $- \triangle - \triangle - \triangle -$ $0.069 \times 10^{-3} M$ of 5-fluorouracil.

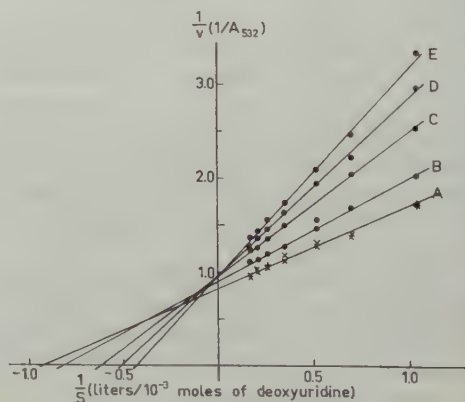


Fig. 4.

Fig. 4. Lineweaver-Burk plot of the inhibition of deoxyuridine-phosphorylase by 5-fluorouracil. Method of assay was deoxyribose determination as described in Experimental. Curve A, no inhibitor (in duplicate); Curve B, $0.06 \times 10^{-3} M$; Curve C, $0.12 \times 10^{-3} M$; Curve D, $0.18 \times 10^{-3} M$ and Curve E, $0.24 \times 10^{-3} M$ of 5-fluorouracil, respectively.

Inhibition of deoxyuridine phosphorylase

Fig. 4 demonstrates the inhibition of deoxyuridine phosphorylase activity by 5-fluorouracil. In the Lineweaver-Burk plot, points of intersection of the curve with the ordinate move upwards from curve A to curves B and C but then remains constant for curves D and E. This means that $V_{\max}$ decreases, when the concentration of 5-fluorouracil rises from 0 to $0.12 \times 10^{-3} M$ but then becomes independent of 5-fluorouracil concentration. At higher 5-fluorouracil concentrations the general pattern of the curves is that of competitive inhibition. Assuming this type of inhibition the calculated K_i -values were $0.50 \times 10^{-3} M$, $0.24 \times 10^{-3} M$, $0.22 \times 10^{-3} M$ and $0.21 \times 10^{-3} M$ for curves B, C, D and E, respectively.

The inhibition of the phosphorolytic cleavage of deoxyuridine-2- ^{14}C by fluorouracil was also studied in a manner similar to that described for uridine-2- ^{14}C cleavage in experiment B of Fig. 1. An inhibition of 50 % was obtained at a 5-fluorouracil concentration of $1.10 \times 10^{-3} M$. A K_i -value of $0.29 \times 10^{-3} M$ was calculated with a K_m -value of $1.10 \times 10^{-3} M$ obtained from Fig. 4.

5-Fluorodeoxyuridine as inhibitor of phosphorolytic cleavage of deoxyuridine-2- ^{14}C was tested in a system identical with that above but with 5-fluorouracil exchanged for 5-fluorodeoxyuridine in final concentrations ranging from $10^{-4} M$ to $5 \times 10^{-3} M$. An inhibition of 50 % was observed at $2.05 \times 10^{-3} M$.

Inhibition of uridine kinase

A few experiments were also performed in order to study the influence of 5-fluorouracil, 5-fluoroorotic acid, 5-fluorodeoxyuridine and 5-fluorouridine on the action of uridine kinase. As assay the isotope method described earlier [13] was used. Of the four substances mentioned only 5-fluorouridine showed an inhibitory effect. Thus 8.5 %, 31.0 % and 56.1 % inhibition was observed at 5-fluorouridine concentrations of $0.5 \times 10^{-3} M$, $5.0 \times 10^{-3} M$ and $7.5 \times 10^{-3} M$, respectively. No effect was observed in similar experiments with 5-fluoroorotic acid, 5-fluorouracil or 5-fluorodeoxyuridine. Lineweaver-Burk plots of data from inhibition experiments with 5-fluorouridine indicated inhibition of a competitive type.

Table 1. Inhibition of uridine phosphorolysis by 5-fluorouracil, 5-fluoroorotic acid and uracil.

The assay of uridine phosphorylase was carried out by incubation at 37° for 15 minutes in a final volume of 0.23 ml. Twenty μ moles of potassium phosphate buffer, pH 7.4, 0.42 μ moles (final concentration = $1.83 \times 10^{-3} M$) of uridine-2- ^{14}C (90,600 c.p.m./ μ mole), extract from 4.5 mg of acetone powder and inhibitors as noted. Analysis by paper chromatography (see Experimental).

	Conc. <i>M</i>	Uracil-2- ^{14}C formed (μ moles)	Inhibition %
Control		0.17	0
5-Fluorouracil	0.43×10^{-3}	0.06	65
	0.87×10^{-3}	0.03	82
	1.30×10^{-3}	0.01	94
5-Fluoroorotic acid	2.18×10^{-3}	0.16	6
Uracil	0.43×10^{-3}	0.13	24
	0.87×10^{-3}	0.12	29
	2.18×10^{-3}	0.08	53

Discussion

The direct utilization of free uracil for nucleotide synthesis was earlier [6, 8] found to be mediated by nucleoside phosphorylases and kinases. This alternative biosynthetic route might be looked upon as a "reserve mechanism" for nucleic acid synthesis in tissues with a high growth rate, e.g. Ehrlich ascites tumor. A rough correlation was found [8] between rate of growth and dependence on this alternative pathway, measured as tissue concentrations of the above enzymes.

Heidelberger *et al.* [4, 5] originally observed that 5-fluorouracil strongly inhibited the incorporation of uracil into RNA in Ehrlich ascites tumor. A preliminary explanation of this was offered by the observation [9] that 5-fluorouracil inhibited uridine phosphorylase activity in a crude extract from this tumor. This has recently been confirmed with whole cells *in vitro* [16].

The aim of the present investigation was primarily to study the enzymic mechanism of this inhibition with a purified enzyme from Ehrlich ascites tumor. The study was also extended to two other purified enzymes of the uracil utilization pathway, deoxyuridine phosphorylase and uridine kinase.

The effect of 5-fluorouracil on uridine phosphorylase was studied in both the direction of synthesis and the direction of cleavage. Inhibition curves (Fig. 1) for the phosphorolytic cleavage of uridine-2-¹⁴C were obtained with crude and purified enzymes and gave identical results. Since the two experiments moreover were performed at different substrate concentrations, the results indicate inhibition of a non-competitive character. This interpretation was confirmed by the pattern of the Lineweaver-Burk curves obtained in the more detailed studies of 5-fluorouracil effect on uridine phosphorolysis performed with the ribose determination method both at pH 7.4 and pH 6.5. The determinations of the enzymic phosphorolysis of uridine thus were based on the formation of uracil-2-¹⁴C in the first case and on ribose-1-phosphate formation in the second. Similar values for K_i were obtained in both cases ($K_i = 0.17 \times 10^{-3} M$ with the isotope method and $K_i = 0.15 \times 10^{-3} M - 0.19 \times 10^{-3} M$ with the ribose method). The observed inhibition is thus not due to a mere trapping of ribose-1-phosphate by formation of 5-fluorouridine [10].

The inhibitory effects on uridine phosphorylase of 5-fluorouridine and uracil, respectively, were about 15 times and 6 times weaker than that of 5-fluorouracil.

The inhibition of uridine formation from uracil and ribose-1-phosphate by 5-fluorouracil was also observed to be of a non-competitive type and the K_i was of the same order of magnitude as that obtained for uridine phosphorolysis.

The Lineweaver-Burk diagrams illustrating the inhibition of deoxyuridine phosphorolysis (Fig. 4) show the general pattern of a competitive inhibition, at least at higher 5-fluorouracil concentrations. At low concentrations of inhibitor, however, the maximum reaction velocity was dependent on the concentration of the inhibitor. At higher concentrations of inhibitor the maximum velocity attained a constant value, which, however, was lower than that observed without inhibitor. The K_i calculations (competitive inhibition) gave identical values ($0.21 \times 10^{-3} M$ to $0.24 \times 10^{-3} M$) for the three curves with a common point of intersection at the ordinate. The above determinations of deoxyuridine phosphorylase activity were based on deoxyribose determinations. When the inhibition of deoxyuridine-2-¹⁴C phosphorolysis was measured by the formation of labelled uracil, a similar K_i value ($0.29 \times 10^{-3} M$) was obtained. This is interpreted to show that the observed inhibition was not due to trapping of deoxyribose-1-phosphate by nucleoside formation with 5-fluorouracil [10].

The specificity of uridine kinase was studied earlier [13]. The enzyme was then shown to phosphorylate 5-fluorouridine but not 5-fluorodeoxyuridine. The affinity of the enzyme was slightly higher for 5-fluorouridine than for uridine. In accordance with this the present investigation showed a competitive inhibition of the phosphorylation of uridine by 5-fluorouridine and no inhibition with 5-fluorodeoxyuridine.

SUMMARY

A study was made of the inhibitory effect of 5-fluorouracil on the enzymes involved in the utilization of uracil for nucleic acid synthesis. 5-Fluorouracil showed a non-competitive inhibition of purified uridine phosphorylase studied both in the direction of uridine phosphorolysis and in the direction of uridine synthesis from uracil + ribose-1-phosphate. An inhibition constant of about 0.16×10^{-3} M was obtained in both cases.

The inhibition of deoxyuridine phosphorylase by 5-fluorouracil measured in the direction of phosphorolysis, was found to be mainly of a competitive type with an inhibition constant of about 0.24×10^{-3} M.

5-Fluoronucleosides showed rather weak inhibitory effects on both phosphorylases.

5-Fluorouridine competitively inhibited uridine kinase, the activity of which was not at all influenced by 5-fluorodeoxyuridine.

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Tryckt den 18 november 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB

Nucleoside and nucleotide derivatives of 5-fluorouracil

Formation with enzymes from Ehrlich ascites tumor

By OLA SKÖLD

With 6 figures in the text

The growth-inhibiting characteristics of the uracil analogue 5-fluorouracil were originally studied by Heidelberger *et al.* [1]. Results from extensive metabolic studies [2–6] have indicated two main mechanisms by which the fluoropyrimidine might exert its growth-inhibiting action: (a) interference with DNA synthesis by the inhibition of thymidylate synthetase thus preventing synthesis of DNA-thymine in the growing cells; (b) formation of a “fraudulent” RNA containing 5-fluorouracil *in lieu* of uracil.

An additional mechanism of growth impairment might be the direct inhibition of uridine phosphorylase by 5-fluorouracil observed earlier [7]. This latter effect prevents the growing cells from utilizing free uracil for RNA synthesis by the alternative pathway for pyrimidine synthesis studied earlier [8].

The two main mechanisms ((a) and (b) above) for the antimetabolite effect of 5-fluorouracil require the transformation of the pyrimidine analogue to the corresponding fluoronucleotides (FUMP and FdUMP).¹ Thus 5-fluorouracil was inactive as an inhibitor of thymidylate synthetase at 500 times the concentration at which inhibition was observed with FdUMP [6]. FdUMP has been found in the acid-soluble fraction from Ehrlich ascites tumor cells incubated *in vitro* with 5-fluorouracil [5]. Furthermore, formation of FUMP can be regarded as a necessary step for the incorporation of 5-fluorouracil into RNA by analogy with the normal RNA-pyrimidine synthesis *via* UMP [9, 10]. This point has been confirmed by the finding of FUMP-2-¹⁴C in the acid-soluble fraction of Ehrlich ascites cells after administration of 5-fluorouracil-2-¹⁴C to tumor-bearing mice [3].

In earlier work [8] Ehrlich ascites tumor was shown to utilize free uracil for RNA synthesis as an alternative route to pyrimidine formation. The tumor was shown to synthesize UMP from uracil by the combined action of uridine phosphorylase and uridine kinase:



¹ The following abbreviations are used in this paper: UMP = uridine-5'-monophosphate, FUMP = 5-fluorouridine-5'-monophosphate, dUMP = deoxyuridine-5'-monophosphate, FdUMP = 5-fluorodeoxyuridine-5'-monophosphate, P_i = inorganic phosphate, ATP = adenosine-5-triphosphate, PGA = 3-phosphoglyceric acid, PCA = perchloric acid, tris = tris(hydroxymethyl)aminomethane-chloride buffer.

Furthermore, relatively high activities of deoxyuridine phosphorylase and deoxyuridine kinase were found in the Ehrlich ascites tumor [11] thus allowing an analogous formation of dUMP:



The two reaction sequences outlined above offer an explanation of the enzymic transformation of 5-fluorouracil to the nucleotide stage. This could thus take place by simple substitution of 5-fluorouracil for uracil in the above reactions. In the present investigation it will be shown that this series of reactions does indeed occur. Part of these findings have been reported earlier [7].

Experimental

Material and methods

5-Fluorouracil, 5-fluorodeoxyuridine and 5-fluorouridine were obtained through the courtesy of the Hoffman-La Roche Co. 5-Fluorouracil-2-¹⁴C was purchased from California Corporation for Biochemical Research. Ribose-1-phosphate was prepared enzymically from inosine with purine nucleoside phosphorylase purified from calf spleen according to Price *et al.* [12]. The xanthine oxidase used to pull the reaction to completion was purchased from Worthington Biochemical corporation. Ribose-1-phosphate was finally precipitated as the barium salt as described by Plesner and Klenow [13]. It showed a purity of 60 % (ribose analysis). The ratio of acid-labile phosphate/ribose was 1:0.85. Before use in enzymic experiments the barium salt was dissolved in water and the barium removed by the addition of a slight excess of Na₂SO₄.

Deoxyribose-1-phosphate was synthesized as the dicyclohexylammonium salt from thymidine with thymidine phosphorylase according to Friedkin [14]. The enzyme preparation¹ was a generous gift from Dr. S. Laland, Biochemistry Department, The University, Oslo. The deoxyribose-1-phosphate obtained showed a purity of 66 % judged from deoxyribose analysis and gave a phosphate/deoxyribose ratio of 1:1.01. When deoxyribose-1-phosphate was kept in aqueous solution, a slightly alkaline pH was maintained.

Separation of 5-fluorouracil and 5-fluorouridine was achieved by starch chromatography with a butanol-water system. Pyrimidine and deoxynucleoside were separated by chromatography on starch in an ammonium acetate-ethanol system as described earlier [11]. Smaller amounts of these fluoro-compounds were separated on paper with a borate solvent [16] and showed the following approximate R_f values: 5-fluorouracil 0.61, 5-fluorouridine 0.23, 5-fluorodeoxyuridine 0.71. These values were determined with the synthetic fluorocompounds obtained from the Hoffman-La Roche Co. Nucleotides were separated by ion exchange chromatography with gradient elution according to Hurlbert *et al.* [17].

Radioactivity was measured by plating on aluminum planchets and counting at infinite thinness in a windowless flow-counter (Tracerlab Sc18).

UV-absorbance determinations were performed with a Beckman DU spectro-

¹ Fraction No. III in the purification procedure of Friedkin and Roberts (15).

photometer. UV spectra were recorded with an automatic Beckman DK2 spectrophotometer.¹

Catalytic hydrogenation of the pyrimidine moiety of 5-fluorouridine and 5-fluorodeoxyuridine was performed according to Cohn and Doherty [18] with a Rhodium catalyst obtained from Baker and Comp., Inc., Newark 5, New Jersey.

Ribose was determined by the method of Mejbaum [19]. Deoxyribose was measured according to Ceriotti [20] or by the method of Waravdekar and Saslaw (21).

Phosphate determinations were performed according to Fiske and SubbaRow [22] and with the Lowry-Lopez [23] method or its micromodification as described by Lowry *et al.* [24].

Most of the experiments were carried out with extracts of acetone powder from Ehrlich ascites tumor. The preparation of acetone powder and its extraction was described earlier [8, 16]. Some experiments were performed with a uridine phosphorylase preparation purified according to Pontis and Reichard [25].

Results

Enzymic formation of 5-fluorouridine

In preliminary experiments the enzymic formation of 5-fluorouridine from 5-fluorouracil and ribose-1-phosphate was observed with an acetone powder extract of Ehrlich ascites tumor. These experiments were performed on a small scale and the incubation mixtures were analyzed by paper chromatography (borate solvent). Spots were observed with an R_f value of approximately 0.23, corresponding to 5-fluorouridine.

In order to obtain a larger amount of the nucleoside analogue 10.0 μ moles of 5-fluorouracil, 50.0 μ moles of ribose-1-phosphate, 100.0 μ moles of tris, pH 7.0, and extract from 138 mg of ascites tumor acetone powder were incubated at 37° for 25 minutes² in a final volume of 3.1 ml. The reaction was stopped by the addition of 0.4 ml of 4 *M* PCA. After 30 minutes at 0° the mixture was centrifuged and the supernatant neutralized (phenol red) with 4 *M* KOH and KClO_4 allowed to precipitate. The supernatant was deionized by consecutive passage through Dowex-2-formate and Dowex-50- H^+ columns (diameter 0.9 cm, length 9 cm, respectively). Each column was eluted with water and the complete elution of the pyrimidine compounds was checked by UV extinction. The final effluent (70 ml) was evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of butanol in water (87:13) and subjected to starch chromatography. When the collected fractions (4–5 ml each) were analyzed by absorbancy measurements at 260 $m\mu$, two distinct peaks were observed. The first one showed an R -value of 1.00 which coincided with that obtained for 5-fluorouracil in a reference chromatogram of the pure substance. The second peak gave an R -value of 0.61. The UV-absorbing substance of this peak was collected by pooling of the appropriate fractions and evaporation to dryness *in vacuo*. The residue was dissolved in water and its UV spectra were recorded at different pH values (Fig. 1).

¹ Kindly placed at my disposal by Prof. B. Malmgren, Department of Bacteriology, Karolinska Institutet, Stockholm.

² With the crude extract as enzyme source the reversible uridine phosphorylase reaction is influenced by phosphatases. In preliminary experiments a time study of the synthesis of uridine-2- ^{14}C from uracil-2- ^{14}C and ribose-1-phosphate was performed. With a fivefold excess of ribose-1-phosphate a maximal amount of uridine-2- ^{14}C was present in the incubation mixture after 20–25 minutes. After this period the uridine-2- ^{14}C concentration declined slowly.

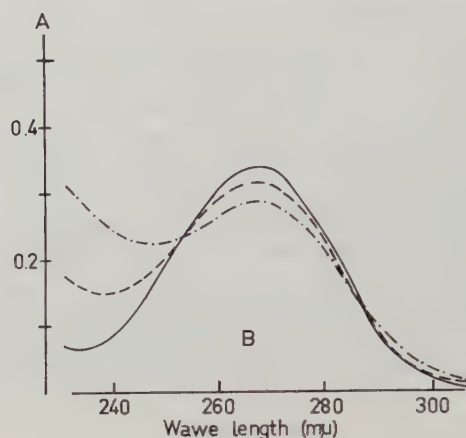


Fig. 1. Ultra-violet absorption spectra of enzymically synthesized 5-fluorouridine (approx. $0.05 \mu\text{mole/ml}$) in 0.1 N HCl —; 0.1 N tris at pH 7.4 ---- and 0.1 N NaOH - · - ·.

These spectra were identical with those obtained for chemically synthesized 5-fluorouridine. The absorption maximum of the enzymically synthesized compounds was $268 \text{ m}\mu$ in both acid and alkali. Its spectrum thus lacked bathochromic shift in alkali shown by 5-fluorouracil, which gave an absorption maximum at $265 \text{ m}\mu$ in acid and at $282 \text{ m}\mu$ in alkali.

The total amounts of 5-fluorouracil and presumptive fluoronucleoside isolated from the starch chromatogram were $5.05 \mu\text{moles}$ and $2.02 \mu\text{moles}$, respectively, based on measurements of UV extinctions.¹

The isolated compound gave a positive orcinol reaction for ribose. This together with the spectrophotometric data of above, identified the substance as the ribonucleoside of 5-fluorouracil. Fig. 2 demonstrates the color development of the orcinol reaction with time for the enzymically synthesized 5-fluorouridine to be much slower than that for uridine.

After catalytic hydrogenation of the pyrimidine moiety, the glycosyl bond between a pyrimidine and ribose is known to be acid-labile [26]. Therefore $0.061 \mu\text{mole}$ of 5-fluorouridine in 1 ml of N HCl was hydrogenated for 1 hour with 2 mg of Rhodium catalyst [18]. After centrifugation of the catalyst ribose determination of the supernatant gave a total of $0.059 \mu\text{mole}$ of ribose. A uridine standard was simultaneously carried through the same procedure.

A time study of the synthesis of 5-fluorouridine from 5-fluorouracil and ribose-1-phosphate was performed with a uridine phosphorylase preparation purified [25] ca. 40 times over a crude acetone powder extract of Ehrlich ascites tumor. As can be seen from Fig. 3 the time curve was linear for more than five hours when ca. 40 % of the added 5-fluorouracil was converted to 5-fluorouridine.

¹ A molar extinction coefficient of 5970 was found for the crystalline 5-fluorouracil at $260 \text{ m}\mu$ and pH 7.4. At this pH the same extinction coefficient was assumed for the nucleoside compound. In acid the corresponding coefficient was found to be 7500.

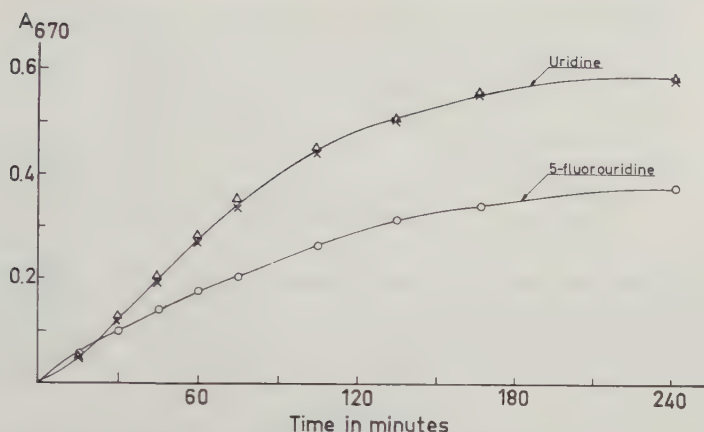


Fig. 2. Color development with time in the orcinol reaction for uridine and 5-fluorouridine. 0.078 μ mole of uridine (in duplicate tubes) and 0.061 μ mole of 5-fluorouridine were heated on a boiling water bath with the reaction mixture of Mejbaum (19). At the time points indicated the mixture was cooled to room temperature and the absorbancy at 670 $m\mu$ was determined.

Enzymic phosphorylation of 5-fluorouridine

The enzymic formation of the nucleotide analogue from 5-fluorouridine was demonstrated by incubating 1.82 μ moles of the enzymically prepared 5-fluorouridine with 100 μ moles of $MgCl_2$, 20 μ moles of ATP, 50 μ moles of PGA, 50 μ moles of tris, pH 7.4, and extract from 138 mg of acetone powder of Ehrlich ascites tumor for 15 minutes at 37° in a final volume of 3.05 ml. The reaction was stopped by the addition of 0.3 ml of 4 *M* PCA. After 1 hour at 0° the supernatant was centrifuged and hydrolysed at 100° for 1 hour to break all pyrophosphate bonds. After neutralization (phenol red) with 4 *M* KOH and sedimentation of precipitated $KClO_4$, the supernatant was chromatographed on Dowex-2 according to Hurlbert *et al.* [17]. An UV-absorbing peak was observed in the chromatogram slightly behind that of UMP. These fractions were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in 1 ml of water. UV data (pH 7.4) showed the formation of 1.68 μ mole of fluoronucleotide, assuming the same molar extinction coefficient as that for 5-fluorouridine.

After catalytic hydrogenation with a Rhodium catalyst, 0.051 μ mole of fluoronucleotide gave 0.051 μ mole of ribose by the orcinol method, with a standard of UMP carried simultaneously through the same procedure.

When 0.012 μ mole of fluoronucleotide was dephosphorylated with a 5'-nucleotidase (Crotalus Adamanteous venom, 7.5 μ g) 0.011 μ mole of inorganic phosphate was liberated.

A direct demonstration of the pyrimidine moiety of the fluoronucleotide was obtained by hydrolysing 0.25 μ mole of nucleotide with PCA according to Marshak and Vogel [27]. After neutralization with KOH paper chromatography in a borate solvent resulted in a single UV spot, corresponding to 5-fluorouracil.

Incubation of 0.18 μ mole of fluoronucleotide with 5'-nucleotidase and subsequent paper chromatography in a borate solvent yielded one spot with an R_f value of that of 5-fluorouridine (0.23).

Table. 1 Analytical data for 5-fluorouridine and FUMP.

	F-uracil μ moles	Ribose μ moles	Phosphate μ moles
5-fluorouridine	1	0.97	—
FUMP	1	1.00	0.92

When the fluoronucleotide was chromatographed on paper in a borate solvent, it hardly moved from the origin (R_f value 0.01).

Table 1 summarizes the analytical data obtained for enzymically synthesized 5-fluorouridine and FUMP.

Enzymic formation of 5-fluorodeoxyuridine

In preliminary experiments with crude acetone powder extracts from Ehrlich ascites tumor it was not possible to observe any deoxynucleoside synthesis from 5-fluorouracil and deoxyribose-1-phosphate, probably because of a rapid destruction of the deoxyribose-1-phosphate by interfering enzymes. Experiments with a dialyzed acetone powder extract, however, showed an extensive cleavage of chemically synthesized 5-fluorodeoxyuridine in the presence of added phosphate, but no such cleavage in the absence of added phosphate. This indicated the presence of a deoxynucleoside phosphorylase. The synthesis of 5-fluorodeoxyuridine from 5-fluorouracil and deoxyribose-1-phosphate was demonstrated with a purified uridine phosphorylase preparation [25] (ammonium sulfate fraction). This was the same enzyme which was used above for the time study of 5-fluorouridine synthesis. Thus 8.0 μ moles of 5-fluorouracil, 15.6 μ moles of deoxyribose-1-phosphate, 100 μ moles of tris, pH 7.4, and 0.08 ml of enzyme preparation were incubated for 3.5 hours at 37° in a final volume of 1.16 ml. The reaction was stopped by the addition of 0.1 ml of 4 *M* PCA. After 30 minutes at 0° the supernatant was neutralized (phenol red) with KOH and the precipitated KClO_4 centrifuged off. An 0.05 ml aliquot of the supernatant was chromatographed on paper and gave two UV-absorbing spots with the same R_f values as 5-fluorouracil and 5-fluorodeoxyuridine. The materials were eluted from the spots and analyzed as described in the legend to Fig. 3. It was found that 76 % of the added 5-fluorouracil was converted to the deoxynucleoside.¹

The remaining supernatant solution was then evaporated to dryness *in vacuo* and the residue was chromatographed on a starch column (4 cm in diameter, 30 cm of length) with ammonium acetate-ethanol, pH 9.5, as described earlier [11]. Two distinct peaks with R -values of 1.2 and 0.69, respectively, were obtained. The materials within each peak were pooled and the solvent was evaporated *in vacuo*.

The material from the first peak was analyzed spectrophotometrically at different pH values. As can be seen from Fig. 4 *A* identical absorption maxima were observed at 268 $m\mu$ in both acid and alkali; thus no bathochromic shift in alkali was present. The spectrum obtained was identical with that of synthetic, crystalline 5-fluorodeoxyuridine. The total amount of 5-fluorodeoxyuridine recovered from the starch

¹ A molar extinction coefficient of 7750 (260 $m\mu$ and pH 2) was calculated for the synthetic, crystalline 5-fluorodeoxyuridine.

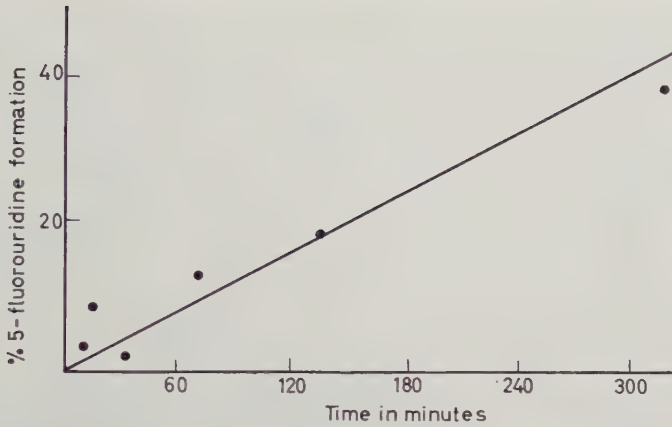


Fig. 3. Time curve for the enzymic formation of 5-fluorouridine from 5-fluorouracil and ribose-1-phosphate with purified uridine phosphorylase. 4 μ moles of 5-fluorouracil, 6 μ moles of ribose-1-phosphate, 16 μ moles of tris, pH 7.4, and 5 μ l of enzyme preparation were incubated at 37° in a final volume of 0.4 ml. At the time points indicated 0.05 ml aliquots were withdrawn and subjected to paper chromatography (see Experimental). The spots corresponding to 5-fluorouracil and 5-fluorouridine were cut out and eluted with water. The amounts of fluorocompounds present were finally measured by UV spectrophotometry.

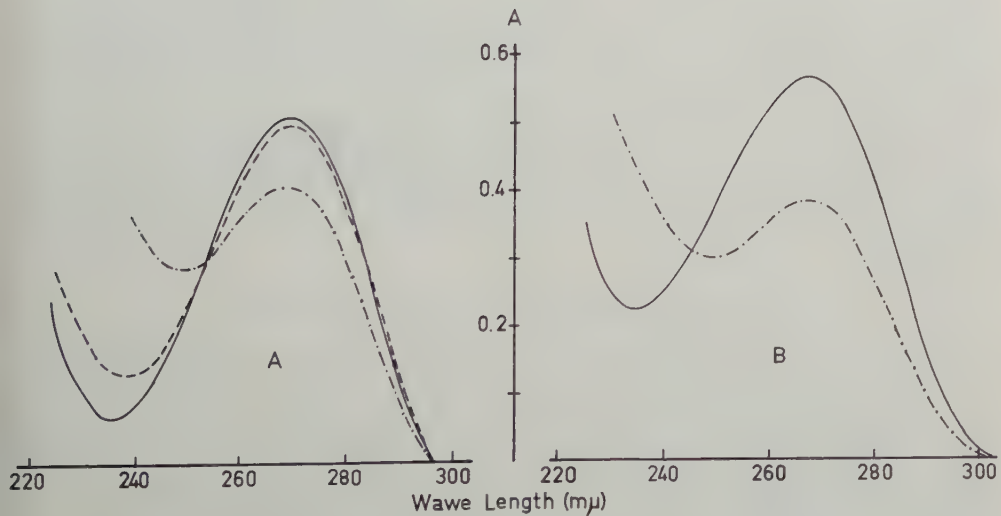


Fig. 4. Ultraviolet absorption spectra. A, enzymically synthesized 5-fluorodeoxyuridine (approx. 0.06 μ mole/ml) in 0.017 N HCl —; 0.017 N tris at pH 7.4. ---- and 0.017 N KOH - · - · -. B, FdUMP and FUMP mixture (approx. 0.07 μ mole/ml calculated for 5-fluorouracil-moiety) in 0.014 M HCl — and 0.014 M KOH - · - · -.

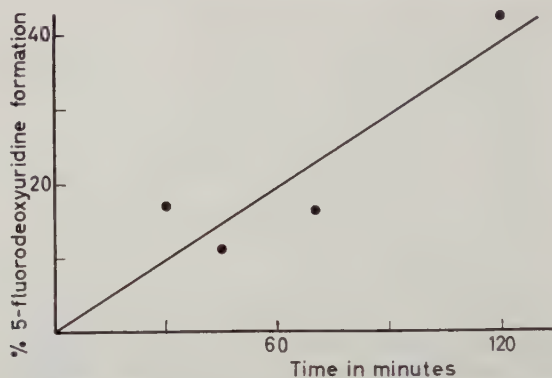


Fig. 5. Time curve for the enzymic formation of 5-fluorodeoxyuridine from 5-fluorouracil and deoxyribose-1-phosphate with purified uridine phosphorylase. Three μ moles of 5-fluorouracil, 5 μ moles of deoxyribose-1-phosphate, 25 μ moles of tris, pH 7.4, and 0.03 ml of enzyme preparation were incubated at 37° in a final volume of 0.31 ml. At the time points indicated aliquots were withdrawn and analyzed as described in the legend to Fig. 3.

column was found to be 4.5 μ moles (UV extinction). The material from the second peak (R-value=0.69) gave ultraviolet spectra identical with those of crystalline 5-fluorouracil. The total amount of 5-fluorouracil recovered from the starch column was 1.5 μ moles (UV extinction). After catalytic hydrogenation performed as described above for 5-fluorouridine, 0.056 μ mole of enzymically synthesized fluorodeoxynucleoside yielded 0.055 μ mole of deoxyribose as determined by the method of Waravdekar and Saslaw [21]. Furthermore, the enzymically synthesized deoxynucleoside migrated exactly as chemically synthesized 5-fluorodeoxyuridine on paper chromatography. Further proof for the identity with 5-fluorodeoxyuridine was obtained by hydrolyzing 1 μ mole of enzymically synthesized compound with PCA according to Marshak and Vogel [27]. On paper chromatography only one spot, corresponding to 5-fluorouracil, was observed.

Fig. 5 shows a time study of the formation of 5-fluorodeoxyuridine from 5-fluorouracil and deoxyribose-1-phosphate with the purified uridine phosphorylase preparation. As shown by the figure, the time curve was linear until about 40 % of 5-fluorouracil was converted to 5-fluorodeoxyuridine, which occurred after about 2 hours of incubation.

Enzymic phosphorylation of 5-fluorodeoxyuridine

The possible enzymic formation of the deoxynucleotide of 5-fluorodeoxyuridine was tested by incubating 3.0 μ moles of 5-fluorodeoxyuridine, 20 μ moles of ATP, 60 μ moles of PGA, 100 μ moles of $MgCl_2$, 100 μ moles of tris at pH 7.4 and a crude extract from 147 mg of acetone powder of Ehrlich ascites tumor at 37° for 15 minutes (final volume = 1.93 ml). After deproteinization with PCA and neutralization with KOH the supernatant was chromatographed on Dowex-2-formate as described by Hurlbert *et al.* [17]. The first UV-absorbing peak coming off the column, as soon as the pH of the effluent turned acid, was shown by paper chromatography to consist of a mixture of 5-fluorouracil and 5-fluorodeoxyuridine. The sample spots were analyzed quantitatively as described in the legend to Fig. 3 and 5-fluorouracil was found to

account for 23.5 % of the fluorouracil-fluorodeoxyuridine mixture. Later in the chromatogram an UV-absorbing peak was found in a position slightly behind that of UMP. The ultraviolet spectra in acid and alkali of this substance were recorded after the pooled fractions had been evaporated to dryness *in vacuo* and redissolved in water. The total amount of material was approximately 0.5 μ mole, when calculated with the same molar extinction coefficient as that of 5-fluorodeoxyuridine. As can be seen from Fig. 4 B the spectra are very similar to those of 5'-fluorodeoxyuridine. A maximum was observed at 267 m μ at both acid and alkaline pH.

When 0.0260 μ mole of the isolated compound was dephosphorylated with 5'-nucleotidase, 0.258 μ mole of inorganic phosphate was liberated. The total material in the corresponding chromatographic peak from a second enzyme experiment was dephosphorylated with 5'-nucleotidase. On paper chromatography two spots were observed, one corresponding to 5-fluorodeoxyuridine, the second corresponding to 5-fluorouridine. The isolated compound thus seemed to be a mixture of the ribonucleotide and the deoxyribonucleotide of 5-fluorouracil.

0.087 μ mole (calculated as described above) of this mixture was dephosphorylated with 5'-nucleotidase and then hydrogenated catalytically as described above for 5-fluorouridine. Subsequent deoxyribose and ribose determination yielded 0.023 μ mole of deoxyribose and 0.064 μ mole of ribose.

The results show that in the second enzyme experiment only about 25 % of the material in the fluoronucleotide peak consisted of the deoxynucleotide.

Formation of 5-fluorouridine phosphates from 5-fluorouridine

During the later stage of this investigation 5-fluorouracil-2-¹⁴C became available. This made possible the enzymic preparation of the labelled ribosyl and deoxyribosyl compounds by exchange reactions between the isotopic fluoropyrimidine and non-labelled fluoronucleosides. The preparations were performed with extracts of *E. coli* essentially as described earlier [11] for the enzymic synthesis of uridine-2-¹⁴C and deoxyuridine-2-¹⁴C. In the present case, however, the labelled nucleosides were isolated by paper chromatography with a borate solvent. The labelled nucleosides were then used for phosphorylation experiments with crude extracts from acetone powder of Ehrlich ascites tumor. Fig. 6 demonstrates such an experiment, where 5-fluorouridine-2-¹⁴C was incubated with ATP and an ATP-regenerating system. Three radioactive peaks appeared in the chromatogram at locations slightly later than uridine-5'-mono-, -di-, and -triphosphate respectively. Most of the radioactivity was recovered in the region of the chromatogram corresponding to a presumptive 5-fluorouridine triphosphate. The material from this peak was hydrolyzed with *N* HCl at 100° for 1 hour, dephosphorylated with 5'-nucleotidase (snake venom) and finally chromatographed on paper with a borate solvent. All of the radioactivity was recovered in one UV spot corresponding to 5-fluorouridine.

When the corresponding phosphorylation experiment was performed with 5-fluorodeoxyuridine-2-¹⁴C three radioactive peaks were obtained with similar locations on the chromatograms as the three peaks observed in the 5-fluorouridine-2-¹⁴C experiment. When the material from the triphosphate peak was dephosphorylated as described above, all radioactivity was recovered in the 5-fluorouridine spot on paper chromatography. The material from the monophosphate peak was also dephosphorylated with 5'-nucleotidase and chromatographed on paper. 78 % of the radioactivity corresponded to 5-fluorodeoxyuridine and 16 % to 5-fluorouridine.

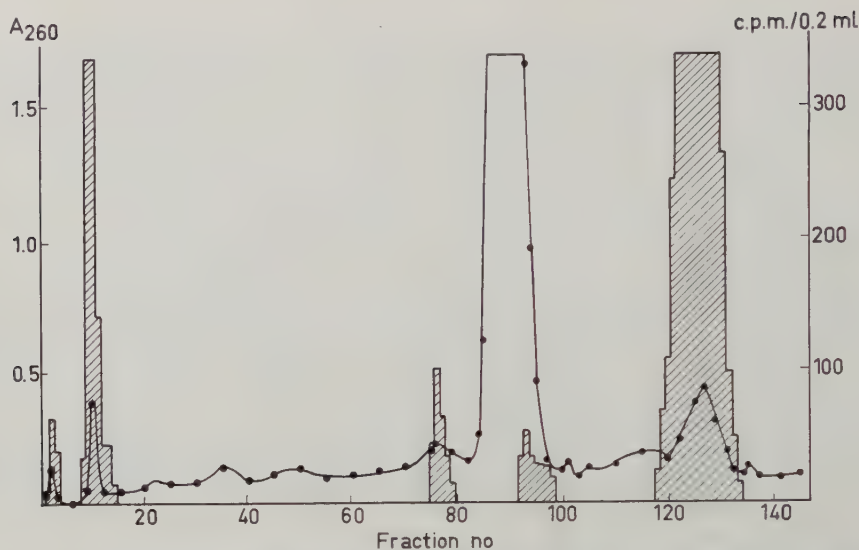


Fig. 6. Formation of 5-fluorouridine phosphates from 5-fluorouridine. The incubation mixture consisted of 1.8 μ moles of 5-fluorouridine-2- 14 C (122,000 c.p.m./ μ mole), 60 μ moles of $MgCl_2$, 10 μ moles of ATP, 20 μ moles of PGA, 50 μ moles of tris, pH 7.4, and extract from 40 mg of acetone powder from Ehrlich ascites tumor (final volume = 2.2 ml). Incubation for 30 minutes at 37°. After deproteinization with PCA and subsequent neutralization the supernatant was subjected to gradient chromatography on Dowex-2-formate according to Hurlbert *et al.* (17). Continuous curve = A_{260} . Stepped curve = counts/0.2 ml/min.

Discussion

In earlier work the biosynthetic utilization of free uracil for nucleotide synthesis has been studied [8, 11, 16]. In general, tissues with a rapid growth rate were found to use this alternative pathway more effectively than tissues with a slow growth rate and this was shown to be due to a higher relative concentration of the nucleoside phosphorylases and kinases responsible for this pathway [11, 16].

Out of a number of mammalian tissues the Ehrlich ascites tumor, which is known for its high growth rate, was thus found to have the highest tissue concentration of the enzymes relevant to this alternative pathway. Enzymes from this tissue was thus chosen for the present investigation, the aim of which was to investigate if the alternative uracil pathway of above is used for the "lethal" synthesis of the nucleotide and deoxynucleotide of 5-fluorouracil. The growth-inhibiting effect of this pyrimidine analogue depends to a large extent on its transformation to the nucleotide stage [3, 5, 6].

Both crude extracts of Ehrlich ascites tumor and a purified preparation of uridine phosphorylase catalyzed the formation of 5-fluorouridine from 5-fluorouracil and ribose-1-phosphate. 5-fluorouridine was identified by the following criteria: (1) the UV spectrum was similar to that of synthetic, crystalline 5-fluorouridine and lacked the bathochromic shift in alkali indicating N_1 -substitution [28]; (2) with the orcinol reaction color development was similar to that for uridine, indicating a similar stability of the glycosyl bond; (3) after catalytic hydrogenation the 5-fluorouracil/ribose ratio was 0.97.

A crude extract of Ehrlich ascites tumor phosphorylated 5-fluorouridine to 5-fluorouridine-5'-monophosphate. The identity of this compound was established by: (1) localization of nucleotide immediately behind the position of UMP on ion exchange chromatography with formate (17); (2) dephosphorylation by a 5'-specific nucleotidase, and extremely slow migration on paper chromatography with a borate solvent establishing the 5'-position of ribose as the point of esterification; (3) a 5-fluorouracil/ribose/phosphate ratio of 1:1.00:0.92 after dephosphorylation and catalytic hydrogenation.

The enzymic synthesis of FUMP in Ehrlich ascites tumor could thus be established according to the following scheme:



The second reaction could also be demonstrated with a purified uridine kinase from Ehrlich ascites tumor (30). When 5-fluorouracil and deoxyribose-1-phosphate were incubated with a uridine phosphorylase preparation from Ehrlich ascites tumor 5-fluorodeoxyuridine was formed. Identification of this compound was based on: (1) the fact that the UV spectrum was identical with that obtained for chemically synthesized 5-fluorodeoxyuridine; (2) deoxyribose determination after catalytic hydrogenation yielded a 5-fluorouracil/deoxyribose ratio of 1:0.98; (3) after acid (PCA) hydrolysis 5-fluorouracil was identified by paper chromatography.

The formation of FdUMP in the presence of a crude acetone powder extract from ascites tumor was expected to be quite small since the deoxyuridine kinase activity is known to be very low (11). After incubation of 5-fluorodeoxyuridine with ATP and crude extract, a mixture of FUMP and FdUMP was found on ion exchange chromatography. FUMP was the major component and was in all probability formed from 5-fluorouracil and ribose-1-phosphate. In the crude extract 5-fluorouracil was supplied by the phosphorolytic cleavage of 5-fluorodeoxyuridine, and ribose-1-phosphate was formed from the added PGA and ATP.

The FUMP-FdUMP mixture showed an UV spectrum quite similar to that reported earlier by Dahl *et al.* (29) for FUMP.

After dephosphorylation with 5'-nucleotidase, 5-fluorouridine and 5-fluorodeoxyuridine were isolated by paper chromatography. A 5-fluorouracil/phosphate ratio of 1:0.99 was obtained for the FUMP-FdUMP mixture.

Ribose and deoxyribose determinations after dephosphorylation and catalytic hydrogenation yielded 0.26 μ mole of deoxyribose and 0.74 μ mole of ribose per μ mole of 5-fluorouracil.

These studies indicate the enzymic formation of FdUMP from 5-fluorouracil and indicate the following scheme for FdUMP synthesis in Ehrlich ascites tumor:



The formation of 5-fluorouridine phosphates with crude extract from Ehrlich ascites tumor shows the same preponderance for the triphosphate as that observed earlier at phosphorylation of uridine (16).

The corresponding phosphorylation experiment with 5-fluorodeoxyuridine-2-¹⁴C

indicated that no pyrophosphates of FdUMP could be formed enzymically. This is in accordance with what is known for dUMP (31). The isolated nucleosidetriphosphate was shown to be a ribosyl compound. Its formation can be explained from the following reaction sequence:



The crude extract of Ehrlich ascites tumor contains all the enzymes necessary for these reactions, which involve the degradation of the deoxynucleoside and a synthesis of the ribonucleoside by nucleoside phosphorylase.

The results of the phosphorylation experiments described above confirm those of Heidelberger *et al.* [3, 5] obtained with whole cells.

SUMMARY

The growth-inhibiting effect of 5-fluorouracil depends to a large extent on its transformation to the nucleotide stage. This transformation was studied with both crude extract and purified enzyme preparations from Ehrlich ascites tumor. Thus uridine phosphorylase catalyzes the formation of 5-fluorouridine from 5-fluorouracil and ribose-1-phosphate and the nucleoside was then phosphorylated to 5-fluorouridine-monophosphate by uridine kinase. Similarly 5-fluorodeoxyuridine was formed from 5-fluorouracil and deoxyribose-1-phosphate with deoxyuridine phosphorylase, and the deoxyribonucleoside could be phosphorylated to 5-fluorodeoxyuridine monophosphate by an extract of Ehrlich ascites tumor. Such an extract also carried out the formation of polyphosphates of 5-fluorouridine but not of 5-fluorodeoxyuridine.

ACKNOWLEDGEMENT

This research was supported by grants from the Swedish Cancer Society, the Damon Runyon Memorial Fund for Cancer Research (Grant No. DRG-470) and from the United States Public Health Service (Grant No. Cy 4619).

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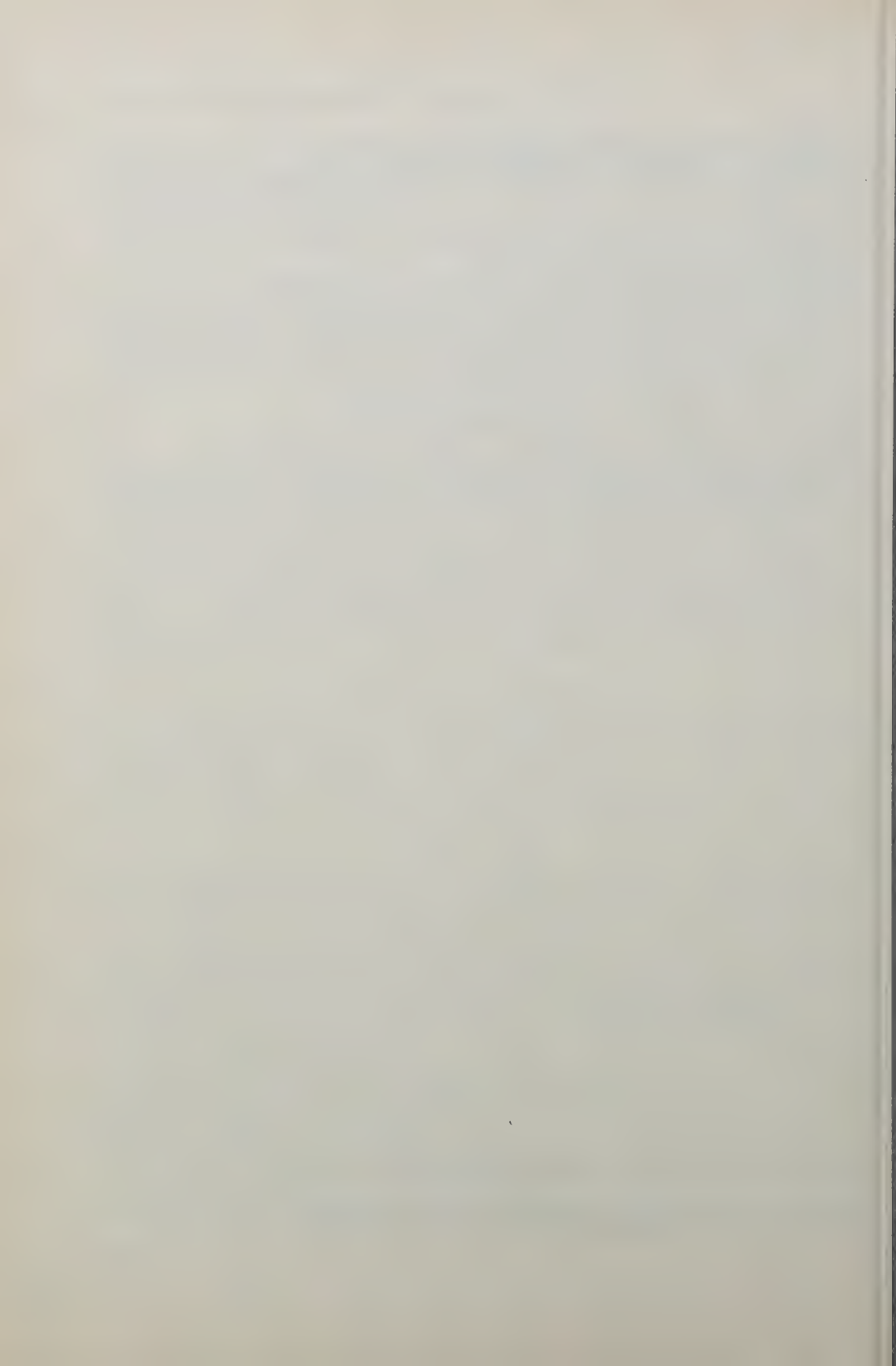
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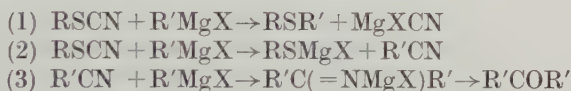
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On the reaction of thiocyanates with organolithium and organomagnesium compounds

By SALO GRONOWITZ and ROLF HÅKANSSON

The literature dealing with the reaction of organic thiocyanates with metalorganic compounds is very scarce. No mention of such reactions occur in Kharasch and Reinmuth's [1] excellent monograph on Grignard reactions of non-metallic substances. The present authors could find only one published investigation, by Adams *et al.* [2], on this subject. These authors reacted excess Grignard reagents (3 moles) with one mole thiocyanate at 0° and found reactions (1), (2) and (3) to have occurred:



Besides the cleavage of the thiocyanates in such a way as to lead to sulphides and inorganic cyanide (1) or to the salts of thiols and organic nitriles (2), the excess of the Grignard reagent used reacts further with the organic nitriles, eventually giving rise to ketones (3). These authors found that with aliphatic Grignard reagents, mixtures of sulphides and thiols were obtained, with aromatic Grignard reagents almost exclusively thiols. In some cases the ketimine hydrochloride or the ketone derived from it were isolated. Data concerning yields or percentage composition of the mixtures obtained were not given. They stated that yields were low when Grignard reagents and thiocyanates of low molecular weight were used, and increased with increasing molecular weight of the reactants. They concluded that the reaction between Grignard reagents and thiocyanates was unsuitable for the preparation of either sulphides or thiols and could hardly be recommended.

In connection with other problems the present authors were interested in a method for preparing sulphides from metalorganic compounds. Although this can be achieved by reacting the metalorganic compound with sulphur followed by an alkyl halide [3], this method has in certain cases several disadvantages. The yields in the reaction with sulphur are sometimes unsatisfactory and only alkyl thioethers can be prepared in this way. Matters are further complicated by the fact that in the preparation of organolithium compounds through halogen-metal interconversion, a halide is formed which competes in the alkylation step for the metalorganic compound [4]. This can in certain cases be an advantage when it is desired to use it as the alkylating agent. A better method giving excellent yields of sulphides is the reaction of metalorganic compounds with disulphides [5]. However, as only half of the disulphide is utilized in the reaction, this may be a severe drawback when the use of more difficultly available disulphides is necessary. As some disulphides are most conveniently prepared from

thiocyanates [6, 7] the direct use of the latter would be advantageous. Aliphatic thiocyanates are easily available from alkyl halides and alkali thiocyanates [8, 9]. Aromatic thiocyanates can be prepared via diazonium salts [9, 10], but especially conveniently and free from isothiocyanates by the aluminium chloride catalysed reaction of aromatics with thiocyanogen, according to the excellent method of Söderbäck [11].

Apart from its possible preparative value, a reinvestigation of the reaction of thiocyanates with metalorganic compounds is also of interest from the point of view of reaction mechanism, giving information about the relative electrophilic reactivity of the sulphur and of the cyano carbon respectively towards the metalorganic compounds.

We have therefore investigated the reaction of thiocyanates with both organomagnesium and organolithium compounds, as characteristic differences in the reactivity of these compounds exist. We have also investigated the temperature effect by carrying out the reaction both at -70° and 0° . The advisability of using low temperature has been demonstrated by Newman & Smith [12] in the preparation of ketones from Grignard reagents and acid anhydrides.

Equimolar quantities of metalorganic compounds and thiocyanates or a slight excess only of the former was used, the former always being added to the thiocyanates. After hydrolyses of the reaction products (with water in the case of organolithium compounds and ammonium chloride solution in the case of Grignard reagents), the thiols formed were extracted with sodium hydroxide solution and, in the appropriate cases, the thiophenol was isolated.¹ The non-acidic products (sulphides and nitriles) formed were, if possible, separated by distillation through a short column as they often had rather different boiling points, and the identity and purity determined by IR-spectroscopy. In some cases close-boiling mixtures of compounds were obtained, the compositions of which were determined directly by IR-analyses.

It was found that at -70° reaction (1) dominated and high yields of sulphides were obtained, especially with aliphatic thiocyanates. The systems investigated and the yields of sulphides obtained are given in Table 1. The yields of nitriles isolated or detected when aliphatic thiocyanates were employed were very low (less than 4%). With phenyl thiocyanate significantly larger amounts of nitriles ($\approx 20\%$) were obtained, yet despite of this the yields of sulphides are still reasonably satisfactory as illustrated by the preparation of 2-thienyl phenyl sulphide and 3-thienyl phenyl sulphide, where yields of 40–50% were obtained. Mixed aromatic aliphatic sulphides are best prepared by reacting aromatic organometallic compounds with aliphatic thiocyanates. Appreciably lower yields are obtained in the reaction between aliphatic metalorganic compounds and aromatic thiocyanates as can be seen in the case of *n*-butyl phenyl sulphide. A significant difference between lithium and Grignard reagents was the appreciably higher yield of benzonitrile obtained in the reaction between phenylmagnesium bromide and phenyl thiocyanate. Although this could be partly due to greater selectivity of the lithium reagents, the fact that organolithium compounds react about 2000 times faster [13] than Grignard reagents with nitriles could also partly account for the lower amount of nitriles isolated when phenyllithium was used. In practice a slight excess of the organolithium compound is usually advisable, especially when the thiocyanate used has similar b.p. as the desired sulphide, in order to avoid contamination with thiocyanates. On the other hand, how-

¹ No attempts were made to determine the amounts of methyl- and butylthiol formed.

Table 1. Yields of sulphides obtained in the reaction between organolithium and organomagnesium compound with thiocyanates at -70° .

Metalorganic compound	Thiocyanate	Sulphide formed	Yield, %
$\text{C}_6\text{H}_5\text{Li}$	$\text{C}_6\text{H}_5\text{SCN}$	$\text{C}_6\text{H}_5\text{SC}_6\text{H}_5$	60
$\text{C}_6\text{H}_5\text{Li}$	$\text{C}_4\text{H}_9\text{SCN}$	$\text{C}_6\text{H}_5\text{SC}_4\text{H}_9$	90
$\text{C}_6\text{H}_5\text{Li}$	CH_3SCN	$\text{C}_6\text{H}_5\text{SCH}_3^a$	73
$\text{C}_4\text{H}_9\text{Li}$	$\text{C}_6\text{H}_5\text{SCN}$	$\text{C}_6\text{H}_5\text{SC}_4\text{H}_9$	45
$\text{C}_4\text{H}_9\text{Li}$	$\text{C}_4\text{H}_9\text{SCN}$	$\text{C}_4\text{H}_9\text{SC}_4\text{H}_9$	87
$\text{C}_4\text{H}_9\text{Li}$	CH_3SCN	$\text{C}_4\text{H}_9\text{SCH}_3$	77
$2\text{-C}_4\text{H}_9\text{SLi}$	$\text{C}_6\text{H}_5\text{SCN}$	$\text{C}_6\text{H}_5\text{-S-(2-C}_4\text{H}_9\text{S)}$	59
$3\text{-C}_4\text{H}_9\text{SLi}$	$\text{C}_6\text{H}_5\text{SCN}$	$\text{C}_6\text{H}_5\text{S-(3-C}_4\text{H}_9\text{S)}$	44
$\text{C}_6\text{H}_5\text{MgBr}$	$\text{C}_6\text{H}_5\text{SCN}$	$\text{C}_6\text{H}_5\text{SC}_6\text{H}_5$	30
$\text{C}_6\text{H}_5\text{MgBr}$	$\text{C}_4\text{H}_9\text{SCN}$	$\text{C}_6\text{H}_5\text{SC}_4\text{H}_9$	79
$\text{C}_6\text{H}_5\text{MgBr}$	CH_3SCN	$\text{C}_6\text{H}_5\text{S-CH}_3^b$	74
$\text{C}_4\text{H}_9\text{MgBr}$	$\text{C}_6\text{H}_5\text{SCN}$	$\text{C}_6\text{H}_5\text{SC}_4\text{H}_9$	56
$\text{C}_4\text{H}_9\text{MgBr}$	$\text{C}_4\text{H}_9\text{SCN}$	$\text{C}_4\text{H}_9\text{SC}_4\text{H}_9$	72
$\text{C}_4\text{H}_9\text{MgBr}$	CH_3SCN	$\text{C}_4\text{H}_9\text{SCH}_3$	87

^a Contains 4%; ^b 3 % of benzonitrile.

ever, excess metalorganic compound should be avoided when the ketone or ketimine formed according to (3) has similar b.p. as the sulphide. Thus in some reactions between phenyllithium and phenyl thiocyanate the sulphide was contaminated with small amounts of benzophenone. In other cases, such as in the reaction between phenylmagnesium bromide and *n*-butyl thiocyanate at 0° , the aromatic ketimines were isolated as such.

The results obtained upon reacting Grignard reagents with thiocyanates at 0° are in general in accordance with the conclusions of Adams *et al.* [2] as to the unsuitability of these reaction conditions for preparative purposes. However, from the point of view of the chemistry of organic thiocyanates, these reactions present some interesting features. From the yields of sulphides and nitriles obtained at the two different temperatures (0° and -70°) it is seen that the relative reaction rate of (2) as compared to (1) increases with increasing temperatures. Thus, for instance, in the reaction between phenylmagnesium bromide and methyl thiocyanate at -70° the yields of thioanisole and benzonitrile obtained were 74 % and 2.5 % respectively, whilst at 0° , 37 % thioanisole and 22 % of benzonitrile was obtained. In the reaction between *n*-butyl thiocyanate and phenylmagnesium bromide at -70° , 78 % of *n*-butyl phenyl sulphide and about 15 % of benzonitrile was obtained, whilst at 0° the yields obtained were 30 % sulphide and 45 % nitrile. The yields of nitrile originally formed are most probably somewhat larger, as some of it can be presumed to have reacted further according to (3), so it should be borne in mind that the yields do not give an exact picture of the relative rates of (1) and (2). That addition to the nitrile formed competes with reaction paths (1) and (2) for the metalorganic reagents is evident from the recovery of thiocyanates in some reactions.

In the reaction between organolithium compounds and thiocyanates, the effect of temperature is not as marked as with the Grignard reagents and good yields of sulphides are still obtained in some cases at the higher temperature. Thus in the reaction between phenyllithium and *n*-butyl thiocyanate at 0° the yield of sulphide

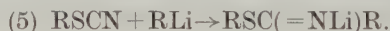
was 79 % and with methyl thiocyanate 68 %. The yields are usually somewhat lower than at -70° and the sulphides sometimes contaminated with ketones. Very little nitriles were isolated in the reaction at 0° , and in some cases even less than at -70° . We ascribe this to the more rapid addition of organolithium compounds to nitriles, which as mentioned before is 2000 times more rapid than with the corresponding Grignard reagents. Thus the amounts of nitriles actually isolated depend upon the relative rates of reaction (1), (2) and (3).

In the reaction between phenyl thiocyanate and metalorganic compounds especially with lithium compounds at 0° diphenyl disulphide is also formed. The amount was so large (in the reaction with *n*-butyllithium 68 %) that it could hardly be due to oxidation of the thiol during the work up, since thiophenol was isolated from the reaction mixture at -70° though the work up in both cases was the same. It is most probable that the mercaptide formed according to (2) reacts with the thiocyanate according to reaction (4)



This reaction is known to take place in alcoholic solutions when *R* is phenyl [14,15]. The difference between lithium compounds and Grignard reagents noticed in this connection might partly be due to differences in solubilities of RSMgX and RSLi . It might be possible to suppress formation of disulphide by the rate of addition of the metalorganic compound thus increasing the yields of sulphides.

We could not isolate any defined products which could have been formed from addition of the metalorganic compound to the $\text{C}\equiv\text{N}$ triple bond in thiocyanates, similar to the reactions of nitriles, giving rise to imino thioethers (5)



We hope to investigate in greater detail the effect of *R* in RSCN on the relative rate of the two types of cleavage that thiocyanates undergo with metalorganic compounds. Our results indicate that characteristic differences exist between aromatic and aliphatic thiocyanates and that the ratio of reaction rates (1) to (2) diminishes in the series $\text{CH}_3 > \text{C}_4\text{H}_9 > \text{C}_6\text{H}_5$.

In a recent investigation, Grant & Snyder [16] reacted 3-thiocyanoindeole with excess ethyl and phenylmagnesium bromide under the same conditions as Adams *et al.* [2] and isolated only 3,3'-diindolyl disulphide and benzonitrile indicating that with thiocyanates having strongly electron-attracting substituents reaction (2) might be dominant.

Adams *et al.* [2] found that the nature of the Grignard reagent had a large influence on the proportion of sulphide and thiol (or nitrile) formed, aromatic Grignard reagents favoring reaction (2). Our results indicate an effect in this direction. However, since this conclusion is based on the amount of nitrile actually isolated, one has to accept it with certain reservations, bearing in mind the complications due to the possibilities of reaction (3).

The fact that the rate of reaction of the metalorganic reagent with the cyano carbon as compared to the sulphur atom is greater in aromatic than aliphatic thiocyanates is rather interesting. Simple reasoning would lead one to expect that the greater electron-attracting properties of a phenyl group as compared to alkyl groups would cause diminished electron-density at the sulphur atom and thus direct the attack of the carbanion of the metalorganic reagent to the sulphur atom. Since this

is not the case, it might be tentatively suggested that the effect of the aromatic group is transmitted to the cyano group across the sulphur bridge. The greater reactivity of a given atom bound through a sulphur atom to an electron-attracting system over that of the sulphur atom in question has been observed by Parker & Kharasch [14, 15] in the cleavage of disulphides with nucleophilic reagents. They interpreted this, however, in terms of thermodynamic control of the cleavage of the disulphides and not to any special relay of electronic effects through the sulphur atom to the adjacent one. However, it is obvious that in our case we must bear in mind that the steric factors as well as the coordination of the metal of the metalorganic compound to the sulphur or to the nitrogen atom would also have considerable influence on the reaction path of thiocyanates. Before any conclusions can be drawn as to the mechanism of the reaction of thiocyanates with metalorganic compounds, the subject has to be studied in much greater detail.

Experimental

Ethereal solutions of phenyllithium [17], *n*-butyllithium [18], phenylmagnesium bromide and *n*-butylmagnesium bromide were prepared under nitrogen according to standard procedures in the usual apparatus, transferred under nitrogen to special burettes and the normality determined in the usual manner by acid titration. Methyl thiocyanate, b.p. 129–130°, $n_D^{20} = 1.4700$ was obtained according to [7] and *n*-butyl thiocyanate, b.p. 66°/11 mm Hg, $n_D^{20} = 1.4645$ according to [9]. Phenyl thiocyanate, b.p. 117–118°/22 mm Hg, $n_D^{20} = 1.5734$ was prepared both according to [9] and [11]. The latter method was the better one, as no phenyl isothiocyanate was formed verified by the absence of its strong characteristic broad band at 4.76μ (2100 cm^{-1}) [19].

Mixtures of sulphides and cyanides or thiocyanates were analysed spectroscopically; calibration curves of the intensities of the cyanide peak at 4.50μ (2222 cm^{-1}) and of the thiocyanate peak at 4.65μ (2150 cm^{-1}) were constructed from known mixtures for this purpose.

A Perkin Elmer model 21 spectrophotometer equipped with sodium chloride prism and a Perkin Elmer Infracord spectrophotometer were used for the IR-analyses.

General procedure in the reaction of thiocyanates and metalorganic compounds

To a solution of 0.1–0.2 moles of the thiocyanate in 150–300 ml of absolute ether in the usual four-necked nitrogen-swept apparatus cooled to -70° in a dry-ice–acetone bath or to 0° in ice-water, was added dropwise during about 1 hr an equivalent amount of an ethereal solution of the metalorganic compound. After standing for an additional hour at the corresponding temperature, water (or ammonium chloride solution in the case of Grignard reagents) was added. When the reactions were carried out at -70° the cooling-bath was removed and the temperature allowed to rise before hydrolysis. The ethereal phases were extracted twice with 2 *N* sodium hydroxide solution in order to remove the thiols. The combined alkaline extracts were acidified and the thiols extracted with ether. When formed, the thiophenol was isolated through distillation. The methylthiol and *n*-butylthiol formed in certain reactions were not isolated. The ether phases containing the non-acidic products were dried over calcium chloride and distilled through a short column.

Reaction between metalorganic compounds and thiocyanates at -70° *A. Organolithium compounds*

Phenyllithium and phenyl thiocyanate. 13.5 g (0.10 moles) of phenyl thiocyanate and 90 ml of 1.15 *N* phenyllithium gave 1.7 g (17 %) of crude benzonitrile, b.p. $81-90^{\circ}/17$ mm Hg, and 11.2 g (60 %) of diphenyl sulphide, b.p. $168-170^{\circ}/25$ mm Hg, $n_D^{20}=1.6298$, whose IR-spectrum was identical with authentic diphenyl sulphide (b.p. $149-151^{\circ}/10$ mm Hg, $n_D^{20}=1.6308$ [20]). 2.1 g (19 %) of crude thiophenol, b.p. $84-86^{\circ}/45$ mm Hg was also obtained.

*Phenyllithium and *n*-butyl thiocyanate.* 15.0 g (0.13 moles) of *n*-butyl thiocyanate and 175 ml of 0.80 *N* phenyllithium gave 16.7 g (77 %) of pure *n*-butyl phenyl sulphide, b.p. $108-112^{\circ}/11$ mm Hg, $n_D^{20}=1.5480$, and 2.8 g (13 %) of slightly less pure *n*-butyl phenyl sulphide, b.p. $112-118^{\circ}/11$ mm Hg, $n_D^{20}=1.5498$, whose IR-spectrum was identical with authentic *n*-butyl phenyl sulphide (b.p. $106-108^{\circ}/11$ mm Hg, $n_D^{20}=1.5475$ [21]). 1.7 g of a fraction, b.p. $118-175^{\circ}/11$ mm Hg, containing besides the above-mentioned sulphide also smaller amounts of benzophenone and benzophenone ketimine was also obtained. No benzonitrile was detected.

**n*-Butyllithium and phenyl thiocyanate.* 13.5 g (0.10 moles) of phenyl thiocyanate and 90 ml of 1.20 *N* *n*-butyllithium gave 1.7 g (20 %) of a fraction, b.p. $66-80^{\circ}/55$ mm Hg, $n_D^{20}=1.4045$ consisting mainly of *n*-butyl cyanide, and 7.5 g (45 %) of *n*-butyl phenyl sulphide, b.p. $109-113^{\circ}/10$ mm Hg, $n_D^{20}=1.5425$. Likewise, 2.0 g of a fraction b.p. $170-180^{\circ}/10$ mm Hg, which crystallized in the condenser and consisted mainly of diphenyl disulphide was obtained. 2.4 g (22 %) of crude thiophenol, b.p. $50-56^{\circ}/10$ mm Hg was obtained from the alkaline extracts.

**n*-Butyllithium and *n*-butyl thiocyanate.* 17.5 g (0.15 moles) of *n*-butyl thiocyanate and 145 ml of 1.15 *N* *n*-butyllithium gave 19.0 g (87 %) of di-*n*-butyl sulphide, b.p. $105-110^{\circ}/55$ mm Hg, $n_D^{20}=1.4525$, whose IR-spectrum was identical with that of authentic di-*n*-butyl sulphide (b.p. $66^{\circ}/10$ mm Hg, $n_D^{20}=1.4539$). 1.2 g of a higher boiling residue was also obtained.

Phenyllithium and methyl thiocyanate. 7.3 g (0.10 moles) of methyl thiocyanate and 167 ml of 0.60 *N* phenyllithium gave 0.5 g (7 %) of recovered methyl thiocyanate b.p. $130-135^{\circ}/760$ mm Hg, 9.5 g of a fraction, b.p. $65-71^{\circ}/10$ mm Hg, $n_D^{20}=1.5802$ which was shown by IR-analysis to consist of 96 % of thioanisole and 4 % benzonitrile.

**n*-Butyllithium and methyl thiocyanate.* 14.6 g (0.20 moles) of methyl thiocyanate and 190 ml of 1.2 *N* *n*-butyllithium gave 16.0 g (77 %) of *n*-butyl methyl sulphide, b.p. $120-125^{\circ}$, $n_D^{20}=1.4453$. (Literature values, b.p. $123-125^{\circ}$, $n_D^{20}=1.4488$.) Its IR-spectrum showed that this fraction contained traces of methyl thiocyanate and *n*-butyl cyanide.

2-Thienyllithium and phenyl thiocyanate. 13.5 g (0.10 moles) of phenyl thiocyanate and an ethereal solution of 2-thienyllithium [22] prepared from 8.5 g (0.10 moles) of thiophene and 82 ml of 1.30 *N* *n*-butyllithium gave 2.1 g (19 %) of crude 2-thienonitrile, b.p. $71-74^{\circ}/11$ mm Hg, $n_D^{20}=1.5545$, identified through IR-comparison with authentic 2-thienonitrile [23]. Likewise, 11.3 g (59 %) of crude 2-thienyl phenyl sulphide, b.p. $95-105^{\circ}/0.4$ mm Hg and 4.7 g of crude diphenyl disulphide b.p. $110-120^{\circ}/0.2$ mm Hg were obtained. Redistillation of the crude 2-thienyl phenyl sulphide gave 8.1 g (42 %) of pure compound, b.p. $93-96^{\circ}/0.3$ mm Hg, $n_D^{20}=1.6485$.

Anal. $C_{10}H_8S_2$ (192.3)

Calc. C 62.46 H 4.19 S 33.35

Found C 62.94 H 4.39 S 33.06

3-Thienyllithium and phenyl thiocyanate. 11.0 g (0.081 moles) of phenyl thiocyanate and an ethereal solution of 3-thienyllithium [24] prepared from 13.2 g (0.081 moles) of 3-bromothiophene [25] and 66 ml of 1.30 *N* *n*-butyllithium gave 2.1 g (24 %) of 3-thienonitrile, b.p. 75–80°/10 mm Hg, $n_D^{20} = 1.5605$, identified through IR-spectrum comparison with authentic 3-thienonitrile [26]. 6.7 g (44 %) of crude 3-thienyl phenyl sulphide b.p. 94–104°/0.4 mm Hg, and 2.0 g of crude diphenyl disulphide b.p. 117–120°/0.4 mm Hg were also obtained. Redistillation of the crude 3-thienyl phenyl sulphide gave 5.2 g (34 %) of the analytically pure compound, b.p. 100–103°/0.3 mm Hg, $n_D^{20} = 1.6520$.

Anal. $C_{15}H_8S_2$ (192.3)

Calc.	C 62.46	H 4.19	S 33.35
Found	C 62.63	H 4.36	S 33.15

B. Organomagnesium compounds

Phenylmagnesium bromide and phenyl thiocyanate. 13.9 g (0.103 moles) of phenyl thiocyanate and 90 ml of 1.25 *N* phenylmagnesium bromide gave 4.2 g (40 %) of a fraction b.p. 66–74°/11 mm Hg consisting mainly of benzonitrile, 1.7 g of a fraction b.p. 74–105°/11 mm Hg consisting of a mixture of benzonitrile and diphenyl sulphide and 5.7 g (30 %) of a fraction of almost pure diphenyl sulphide, b.p. 145–160°/11 mm Hg. The residue consisted mainly of diphenyl disulphide. 2.7 g (25 %) of crude thio-phenol, b.p. 53–57°/12 mm Hg was also obtained.

*Phenylmagnesium bromide and *n*-butyl thiocyanate.* 17.0 g (0.148 moles) of *n*-butyl thiocyanate and 125 ml of 1.19 *N* phenylmagnesium bromide gave 3.2 g (20 %) of a fraction, b.p. 70–110°/11 mm Hg. IR-spectroscopy showed this to be mainly benzonitrile with smaller amounts of *n*-butyl thiocyanate and *n*-butyl phenyl sulphide. The main fraction, 19.5 g (79 %), b.p. 110°/11 mm Hg, $n_D^{20} = 1.5474$, was shown by IR-analysis to be pure *n*-butyl phenyl sulphide.

**n*-Butylmagnesium bromide and phenyl thiocyanate.* 13.5 g (0.10 moles) of phenyl thiocyanate and 100 ml of 1.08 *N* *n*-butylmagnesium bromide gave 0.8 g (10 %) of crude *n*-butyl cyanide, b.p. 55–65°/50 mm Hg, $n_D^{20} = 1.4093$, and 9.3 g (56 %) of pure *n*-butyl phenyl sulphide, b.p. 134–138°/30 mm Hg, $n_D^{20} = 1.5474$. A residue of 1.2 g consisting mainly of diphenyl disulphide was also obtained.

**n*-Butylmagnesium bromide and *n*-butyl thiocyanate.* 19.5 g (0.17 moles) of *n*-butyl thiocyanate and 160 ml of 1.1 *N* *n*-butylmagnesium bromide gave 17.6 g (72 %) of di-*n*-butyl sulphide, b.p. 86–88°/25 mm Hg, $n_D^{20} = 1.4552$, containing traces of *n*-butyl thiocyanate.

Phenylmagnesium bromide and methyl thiocyanate. 11.6 g (0.159 moles) of methyl thiocyanate and 150 ml of 1.15 *N* phenylmagnesium bromide gave 15.0 g of a fraction, b.p. 69–73°/11 mm Hg, $n_D^{20} = 1.5795$ which was shown by IR-analyses to consist of 97 % thioanisole and 3 % benzonitrile.

**n*-Butylmagnesium bromide and methyl thiocyanate.* 14.5 g (0.20 moles) of methyl thiocyanate and 185 ml of 1.1 *N* *n*-butylmagnesium bromide gave 18.0 g (87 %) of pure *n*-butyl methyl sulphide, b.p. 119–122°, $n_D^{20} = 1.4452$.

Reactions between metalorganic compounds and thiocyanates at 0°

A. Organolithium compounds

Phenyllithium and phenyl thiocyanate. 15.0 g (0.111 moles) of phenyl thiocyanate and 120 ml of 0.95 *N* phenyllithium gave 1.5 g of a fraction b.p. 71–143°/11 mm Hg consisting of impure benzonitrile followed by 8.3 g (40 %) of impure diphenyl sulphide,

b.p. 143–155°/11 mm Hg, containing smaller amounts of benzophenone and diphenyl disulphide. 3.0 g of a fraction, b.p. 155–190°/11 mm Hg and 2.5 g of a residue consisting mainly of the three last-mentioned compounds was also obtained. From the alkaline extracts 2.5 g (20 %) of crude thiophenol, b.p. 54–58°/11 mm Hg was obtained.

Phenyllithium and n-butyl thiocyanate. 15 g (0.13 moles) of *n*-butyl thiocyanate and 140 ml of 0.95 *N* phenyllithium gave 1.3 g of a fraction b.p. 78–95°/11 mm Hg which consisted of about equal amounts of benzonitrile and *n*-butyl phenyl sulphide and 17.0 g (79 %) of almost pure *n*-butyl phenyl sulphide, b.p. 105–110°/11 mm Hg, $n_D^{20} = 1.5410$.

n-Butyllithium and phenyl thiocyanate. 13.5 g (0.10 moles) of phenyl thiocyanate and 92 ml of 1.15 *N* *n*-butyllithium gave 1.8 g (21 %) of a fraction b.p. 55–60°/55 mm Hg, consisting mainly of *n*-butyl cyanide, 3.5 g (21 %) of a fraction, b.p. 126–136°/19 mm Hg consisting mainly of *n*-butyl phenyl sulphide and 7.5 g (69 %) of diphenyl disulphide, b.p. 195–198°/19 mm Hg, m.p. 54–58°.

n-Butyllithium and n-butyl thiocyanate. 18.0 g (0.156 moles) of *n*-butyl thiocyanate and 150 ml of 1.15 *N* *n*-butyllithium gave 13.2 g (58 %) of di-*n*-butyl sulphide, b.p. 105–110°/55 mm Hg, $n_D^{20} = 1.4535$. This contained traces of dibutyl ketone. 1.9 g of a fraction, b.p. 105–125°/10 mm Hg was also obtained.

Phenyllithium and methyl thiocyanate. 14.0 g (0.19 moles) of methyl thiocyanate and 205 ml of 0.95 *N* phenyllithium gave 16.5 g of a fraction, b.p. 72–76°/12 mm Hg, $n_D^{20} = 1.5846$, which consisted of 98 % thioanisole and 2 % benzonitrile. 2.8 g of residue was also obtained.

n-Butyllithium and methyl thiocyanate. 14.6 g (0.200 moles) of methyl thiocyanate and 186 ml of 1.25 *N* *n*-butyllithium gave 10.7 g (51 %) of impure *n*-butyl methyl sulphide, b.p. 119–129°, $n_D^{20} = 1.4468$. 2.0 g of residue contained some methyl thiocyanate and dibutyl ketone.

B. Organomagnesium compounds

Phenylmagnesium bromide and phenyl thiocyanate. 14.0 g (0.104 moles) of phenyl thiocyanate and 88 ml of 1.19 *N* phenylmagnesium bromide gave 2.0 g (19 %) of almost pure benzonitrile, b.p. 70–74°/12 mm Hg. 2.0 g of a fraction, b.p. 74–140°/12 mm Hg, consisting of a mixture of benzonitrile, phenyl thiocyanate and some diphenyl sulphide, as well as 5.2 g (27 %) of impure diphenyl sulphide, b.p. 145–160°/12 mm Hg, $n_D^{20} = 1.6252$, containing benzophenone and diphenyl disulphide was obtained. The residue weighed 3.5 g. 2.0 g (17 %) of impure thiophenol was recovered from the alkaline extract.

Phenylmagnesium bromide and n-butyl thiocyanate. 17.0 g (0.15 moles) of *n*-butyl thiocyanate and 125 ml of 1.19 *N* phenylmagnesium bromide gave 4.8 g (31 %) of a fraction, b.p. 69–75°/11 mm Hg, consisting mainly of benzonitrile and some *n*-butyl thiocyanate and 2.8 g of a fraction, b.p. 75–105°/11 mm Hg, being a mixture of benzonitrile and *n*-butyl phenyl sulphide. 7.6 g (30 %) of impure *n*-butyl phenyl sulphide, b.p. 110–115°/11 mm Hg and 3.5 g of a fraction, b.p. 160°/11 mm Hg, $n_D^{20} = 1.6130$, which evolved ammonia on standing and which proved to be a mixture of benzophenone and benzophenone ketimine was also obtained. The ketimine was identified through the characteristic =NH stretching at 3.09μ (3236 cm^{-1}) and the C=N stretching at 6.24μ (1602 cm^{-1}) [27].

n-Butylmagnesium bromide and phenyl thiocyanate. 13.5 g (0.10 moles) of phenyl

ithiocyanate and 100 ml of 1.06 *N* *n*-butylmagnesium bromide gave 1.2 g (14 %) of mpure *n*-butyl cyanide, b.p. 60–65°/50 mm Hg and 7.5 g of a fraction, b.p. 130–138°/30 mm Hg which consisted of 77 % of *n*-butyl phenyl sulphide and 23 % of phenyl thiocyanate. 2.2 g (20 %) of thiophenol, b.p. 56–60°/12 mm Hg was also obtained.

n-Butylmagnesium bromide and *n*-butyl thiocyanate. 19.5 g (0.17 moles) of *n*-butyl thiocyanate and 160 ml of 1.10 *N* *n*-butylmagnesium bromide gave 16.0 g of a fraction, b.p. 65–125°/55 mm Hg, which consisted of a mixture of about 90 % of di-*n*-butyl sulphide and 10 % of *n*-butyl thiocyanate. 4 g of a residue was obtained.

Phenylmagnesium bromide and methyl thiocyanate. 12.0 g (0.165 moles) of methyl thiocyanate and 160 ml of 1.15 *N* phenylmagnesium bromide gave 11.2 g of a fraction, b.p. 68–72°/10 mm Hg. $n_D^{20} = 1.5655$, which consisted of a mixture of 33 % of benzonitrile and 67 % of thioanisole. 3 g of a fraction, b.p. 125–130°/0.5 mm Hg consisted of a mixture of benzophenone and benzophenone ketimine.

n-Butylmagnesium bromide and methyl thiocyanate. 14.5 g (0.20 moles) of methyl thiocyanate and 185 ml of 1.1 *N* *n*-butylmagnesium bromide gave 15.1 g of a fraction, b.p. 115–135° which consisted of 82 % of *n*-butyl methyl sulphide and 18 % of methyl thiocyanate.

SUMMARY

The reaction of organolithium or organomagnesium compounds with organic thiocyanates at -70° leads predominantly to the formation of sulphides. With aliphatic thiocyanates, yields between 70–90 % were obtained, with phenyl thiocyanate the yields were between 40–60 %. 2-Thienyl phenyl sulphide and 3-thienyl phenyl sulphide have been prepared from the isomeric thienyllithium and phenyl thiocyanate.

At 0° , and especially with Grignard reagents, the cleavage of thiocyanates leading to nitriles and the salt of thiols is more marked than at -70° and mixtures of sulphides, thiols and nitriles and secondary reaction products are formed.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Prof. Arne Fredga for his interest in this work and for all facilities put at their disposal. Thanks are also due to Dr. E. Söderbäck for placing phenyl thiocyanate at our disposal and for many interesting discussions on the chemistry of thiocyanogen and thiocyanates. Thanks are also due to Mr. S. Johansson for recording some of the IR-spectra and to Mr. F. Lövgren for the preparation of methyl thiocyanate and diphenyl sulphide. The elementary analyses were carried out at the Analytical Department of this Institute. A grant from the Swedish Natural Science Research Council to S.G. is gratefully acknowledged.

Institute of Chemistry, University of Uppsala, Sweden, September 1960.

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Tryckt den 7 december 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB

N-acylierte Pyrazolone

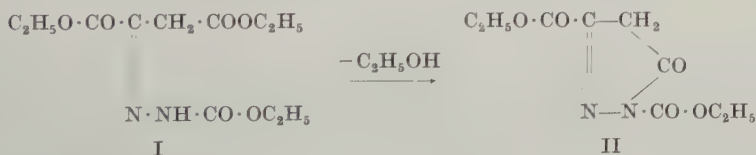
Von ERIK WAHLBERG

Mit 4 Figuren im Text

Für die Herstellung der Pyrazolone, die am N¹ acyliert sind, habe ich die entsprechenden Hydrazone als Ausgangsmaterial gewählt.

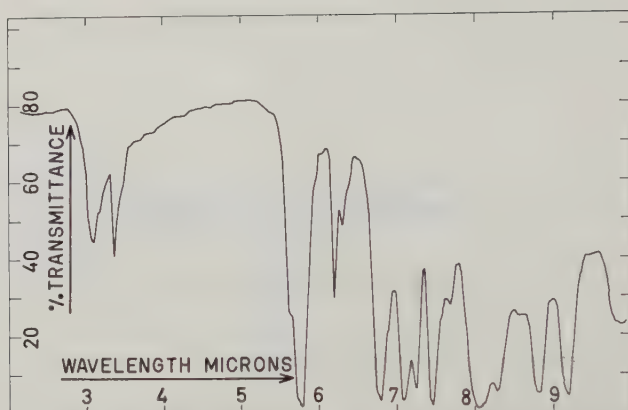
Um aus α -Oxalessigsäureäthylestercarbäthoxyhydrazon [1] *Pyrazolon-(5)-dicarbonsäure-(1.3)-diäthylester* herzustellen, wurden zahlreiche Versuche gemacht. Erwärmung des Hydrazons im Vakuum bei einer Temperatur über dem Schmelzpunkt oder Erwärmung in einem Lösungsmittel z. B. Wasser, Eisessig, Isopropyläther verursachte Abspaltung der am N gebundenen Carbäthoxylgruppe. Auch konnte eine Umwandlung von α - zu β -Hydrazon beobachtet werden. Denselben Erfolg hatte eine Behandlung mit alkoholischer Schwefelsäure und mit gesättigter Sodalösung. In diesen Fällen wurden etwa gleiche Teile α - und β -Hydrazon als Hauptprodukt neben etwas Zersetzungsprodukten, wie Pyrazolon-(5)-carbonsäure-(3)-äthylester, isoliert. Bei der Behandlung mit Natriumalkoholat in alkoholischer Lösung wurde kaum mehr als das letzterwähnte Pyrazolon erhalten.

Das β -Hydrazon scheint also im allgemeinen bei den Reaktionen durch Umlagerung gebildet zu werden. Wenn man α -Hydrazon I in etwa 1 *m* Sodalösung auflöst, erhält man eine anfänglich farblose Lösung, die sich schnell gelb färbt. Aus dieser Lösung lässt sich etwas Pyrazolondicarbonsäurediäthylester II als farblose Kristalle isolieren.



Eine bessere Ausbeute wurde aber erhalten, wenn das Ammoniumsalz des Pyrazolons mittels trockenen Ammoniaks aus einer Lösung von α -Hydrazon in einer Mischung von Äthanol und Äther gefällt und daraus das Pyrazolon freigemacht wurde.

Nahezu quantitativ wurde die Ausbeute bei der Ausführung der Reaktion in dem heterogenen System Wasser und Äther mittels Natriumcarbonat. Der Hintergrund dieses guten Resultates waren die verschiedenen Reaktionsgeschwindigkeiten in Wasser und feuchtem Äther. Eine Lösung von α -Hydrazon in trockenem Äther scheint unbegrenzt und in feuchtem Äther mehrere Wochen haltbar zu sein. In Wasser löst sich α -Hydrazon zu knapp 0,5 %. Erst nach einem Tag kann eine

Abb. 1. IR-Spektrum des Pyrazolon-(5)-dicarbonsäure-(1.3)-diäthylesters in 10 % CCl_4 -Lösung.

Bildung von Pyrazolon durch eine unscheinbare Färbung mit FeCl_3 nachgewiesen werden. Nach Eintrocknen bei Zimmertemperatur wurde ein Kristallkuchen erhalten, der überwiegend α -Hydrazon enthielt, und dazu etwas gelbes Öl, aus Pyrazolon und Zersetzungsprodukten bestehend. Ich konnte kein β -Hydrazon nachweisen.

Das Pyrazolon II löst sich farblos und bis zu etwa 5 % in Wasser. In Wasserlösung wird das Pyrazolon langsam, in alkalischer Lösung aber ziemlich schnell zersetzt. Eine Sodalösung des Pyrazolons II muss also schleunigst neutralisiert werden.

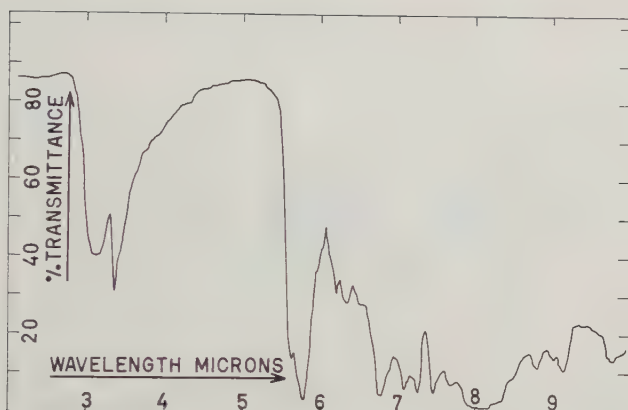
Bei Destillation des Hydrazons im Hochvakuum geht die Hauptmenge bei 1,5 mm und 188–190° unter Zersetzung über. Aus dem erhaltenen Öl, in Petroläther gelöst, konnte nahezu reines β -Hydrazon mit Schmelzpunkt 35–37° durch Ausfrieren isoliert werden. Der Rückstand war ein Öl, welches ausser etwas β -Hydrazon wahrscheinlich ein Pyrazolon enthielt. Darum wurden die IR-Spektren dieses Öls und des Pyrazolons II, beide in CCl_4 gelöst, verglichen. Abb. 1 und 2.

Tabelle 1.

Absorptionb. in μ : a = Pyrazolon; b = Dest. pr.										
(a)	3,30	3,36	5,62	5,78	6,20	6,30	6,80	7,09	7,25	7,47
(b)	3,15	3,36	5,62	5,78	6,20	6,30	6,80	7,08	7,25	7,45
(a)	7,68	8,02	8,8	9,2						
(b)	7,68	8,02	8,8	9,0						

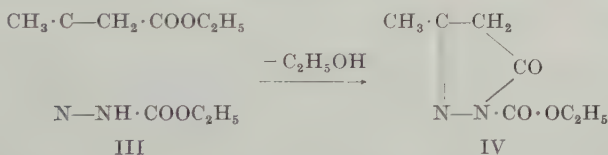
Die IR-Spektren zeigen, dass in dem Destillationsprodukt das Pyrazolon II und nicht ein Isomeres vorliegen muss.

Etwas mehr vom erwähnten Öl wurde bei wiederholten Destillationen bei 0,1 mm erhalten (ca 10 % des Materials), aus welchem es mir gelungen ist, Kristalle von dem Schmelzpunkt des Pyrazolons II zu isolieren.


 Abb. 2. IR-Spektrum des Destillationsprodukts in 10 % CCl_4 -Lösung.

3-Methyl-5-pyrazolon-1-carbonsäureäthylester wurde angeblich von Backer und Meyer [2] durch Behandlung von 3-Methyl-5-pyrazolon mit Chlorameisensäureäthylester erhalten. Das Produkt schmolz bei 203° und die Wasser- oder Alkohollösung wurde von FeCl_3 rotbraun gefärbt. Sie haben die Kondensation zwischen Acetessigsäureester und Hydrazincarbonensäureäthylester vermieden, weil die Carbäthoxylgruppe losbrechen konnte.

Ich habe die Kondensation mit Hydrazincarbonensäureäthylester bevorzugt und dabei erst das Hydrazon III [1] isoliert, um dann dieses in Pyrazolon umzuwandeln. Dabei habe ich auch ein anderes Produkt erhalten.



Meyer hatte bei Erhitzung dieses Hydrazons III im Vakuum bei 110° das oben erwähnte Pyrazolon vom Schmelzpunkt 203° erhalten. Bei meiner Ausführung dieser Operation bei 70 – 80° wurde eine Kristallmischung erhalten, aus welcher gelbe Kristalle vom Schmelzpunkt 242 – 243° isoliert wurden. Diese wurden von FeCl_3 blauviolett gefärbt. Vielleicht war es dieselbe Verbindung vom Schmelzpunkt 246° , die Rosengarten [3] bei Erhitzung von Hydrazin und überschüssigem Acetessigester erhielt. Es gelang mir nicht ein Pyrazolon vom Schmelzpunkt 203° zu isolieren, wohl aber ein Pyrazolon mit Schmp. 136° .

Das Hydrazon III konnte in der Kälte mit Natriumäthylat ohne eine Abspaltung der Carbäthoxylgruppe behandelt werden. Das dabei gebildete Na-Salz des Pyrazolons IV wurde mit quantitativer Ausbeute isoliert. Die daraus freigemachten gelblichen Kristalle des Pyrazolons schmelzen bei 136 – 136.5° und geben mit FeCl_3 eine blauviolette Farbe.

Die Darstellungsweise führt zu einer Konstitution nach Formel IV. Das von Meyer angegebene Pyrazolon muss eine andere haben, wahrscheinlich nach Formel V

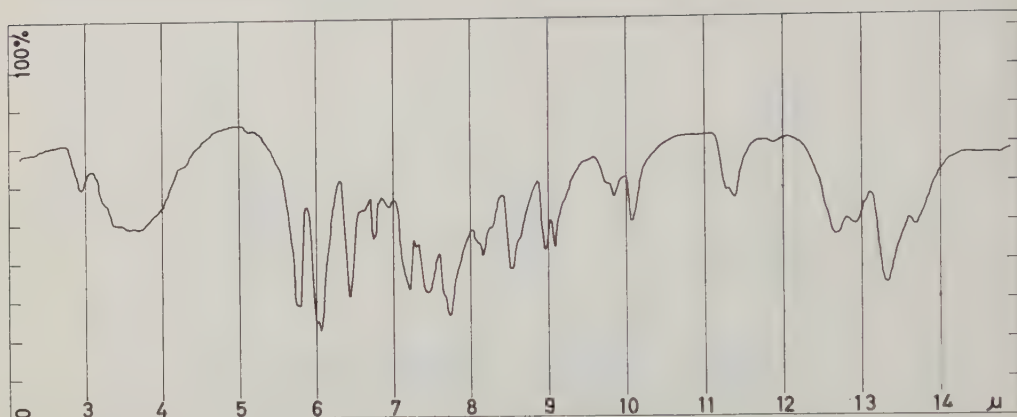
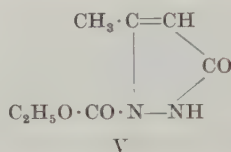


Abb. 3. IR-Spektrum des 3-Methyl-5-pyrazolon-1-carbonsäureäthylesters.



Das NH_4 -Salz des Pyrazolon IV wurde auf die vorher bei Pyrazolon II erwähnte Weise hergestellt. Wenn es in alkoholischer Lösung gekocht wurde, verlor es Ammoniak und Carbäthoxylgruppe. Das daraus isolierte Pyrazolon schmolz bei 215° und gab dieselbe Analysendaten wie Methylpyrazolon. Meyer hatte das Pyrazolon mit Schmelzpunkt 203° erhalten.

Es ist mir nicht gelungen, durch Erwärmung oder auf andere Weise Hydrazon III oder Pyrazolon IV in das von Meyer aus Methylpyrazolon dargestellte Pyrazolon mit Schmelzpunkt 203° überzuführen. Wohl aber konnte ich Methylpyrazolon aus dem Reaktionsprodukt isolieren, welches also durch Zersetzung entstanden war. Die Schmelzpunkte geben wenig sicheren Anhalt für die Beurteilung. Die betreffenden Substanzen sind sehr empfindlich für winzige Verunreinigung. Das von mir nach Meyers Beschreibung aus Methylpyrazolon dargestellte Pyrazolon schmolz nach Umkristallisieren aus Alkohol bei $206,5^\circ$ – 207° und zeigte dabei völlig richtige Analyse ($\text{N} = 16,47\%$). Die in der Literatur für Methylpyrazolon angegebenen Schmelzpunkte variieren erheblich. Dieses Pyrazolon wird in alkohol. Lösung von FeCl_3 rotbraun gefärbt.

Beim Vergleich der IR-Spektren der beiden Pyrazolone sieht man, dass sie nicht identisch sind. Das Band der CO-Valenzschwingung hat für beide nahezu identische Lage. Das Meyer-Pyrazolon (Abb. 4) zeigt eine Reihe von Bändern (6,26; 6,58; 6,68; 7,14; 7,53; 7,78; 9,60 usw.), für welche keine entsprechenden Werte bei dem anderen Pyrazolon zu finden sind.

Versuche

Pyrazolon-(5)-dicarbonsäure-(1.3)-diäthylester wird am besten durch Einwirkung von verdünnter Sodalösung auf Hydrazon I dargestellt. Eine bei Zimmertemperatur

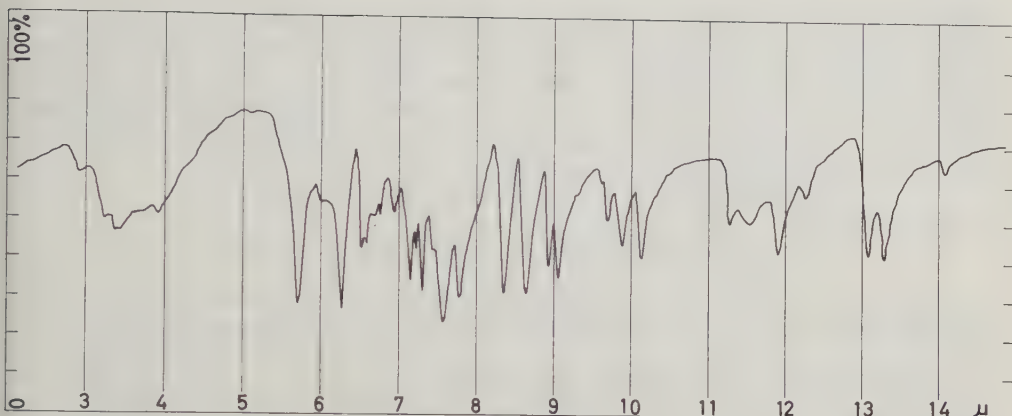


Abb. 4. IR-Spektrum des nach Meyer dargest. Pyrazolons.

gesättigte Lösung von 10 g Hydrazon I in 450 ml Äther (ca. 3 %) wird zweimal 10 Min. mit jedesmal 150 ml gekühlter 0,25 *m* Sodalösung durchgeschüttelt. Jede Sodalösung wird sofort mit 38 ml 1 *m* H₂SO₄ sauer gemacht. Die Pyrazolonlösung wird mehrmals mit etwa 200 ml Äther ausgesogen und die Ätherlösung mit Na₂SO₄ kurze Zeit getrocknet und bei tiefer Temperatur, am besten im Vakuum, eingedampft. Nach Umkristallisieren aus Petroläther schmilzt das Pyrazolon bei 86–86,5°.

C ₉ H ₁₂ O ₅ N ₂ (228,23)	Ber.	C 47,36	H 5,30	N 12,22
	Gef.	C 47,36	H 5,28	N 12,14

Das Pyrazolon ist leicht löslich in Äther, Tetrachlorkohlenstoff, Benzol, Wasser, Alkohol und Aceton, schwer in Benzin und Petroläther.

NH₄-Salz des Pyrazolon-dicarbonsäure-diäthylesters: Eine bei 20° gesättigte Lösung von 13,7 g (0,05 Mol) des Oxalessigester-carbäthoxyhydrazon I in 137 ml 99,9 % Äthanol (11,2 %) wird mit 100 ml trockenem Äther verdünnt. Unter Umrühren und Kühlung wird trocknes Ammoniak in die Lösung geleitet. Die Mischung wird bald von farblosen Kristallen getrübt. Sobald kaum mehr NH₃ absorbiert wird, verdünnt man mit etwa 5 Teilen Äther und kühlt in Kältemischung. Die abgesaugten Kristalle werden mit gekühltem Äther gewaschen. Ausbeute 47 % d. Th. Durch schnelle Auflösung in Äthylalkohol und rasche Abkühlung in Kältemischung kann das NH₄-Salz umkristallisiert werden. Schmp. 128–129° unter Zersetzung.

C ₉ H ₁₅ O ₅ N ₃ (245,24)	Ber.	N 17,14
	Gef.	N 17,12

Aus einer Wasserlösung des NH₄-Salzes erhält man mit verd. H₂SO₄ in Anwesenheit von Äther das Pyrazolon, aber ein Teil wird dabei zersetzt unter Bildung von Pyrazolon-monocarbonsäureäthylester.

3-Methyl-5-pyrazolon-1-carbonsäurediäthylester: Eine bei Zimmertemperatur gesättigte Lösung von 10 g Acetessigsäureäthylester-carbäthoxyhydrazon III in 36 ml 99,5 % Äthanol (26,03 %) wird unter Kühlung mit einer Natriumäthylatlösung von 1,1 g Na in 20 ml Äthanol versetzt. Die Mischung bleibt einige Stunden unter Kühlung

stehen. Nun wird mit 400 ml in Kältemischung gekühltem Äther verdünnt und das ausgefallene farblose Salz abgesaugt. Noch etwas mehr kann beim Verdampfen des Äthers erhalten werden. Das Salz wird mit gekühlter 0.2 *m* H₂SO₄ (etwa 140 ml) digeriert und die gelblichen Kristalle sofort abgesaugt. Nach Umkristallisieren aus Wasser oder Essigester schmilzt das Pyrazolon bei 136–136,5°. Die alkoholische Lösung wird von FeCl₃ blauviolett gefärbt.

C ₇ H ₁₀ O ₃ N ₂ (170,17)	Ber.	C 49,40	H 5,92	N 16,47
	Gef.	C 49,19	H 5,97	N 16,27

*NH*₄-Salz des 3-Methyl-5-pyrazolon-1-carbonsäureäthylesters wurde auf die oben erwähnte Weise hergestellt. Die Ausbeute war 60 % d. Th. Schmp. 163–164°.

C ₇ H ₁₃ O ₃ N ₃ (187,20)	Ber.	N 22,45	Gef.	N 22,03
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Herrn Professor Arne Fredga, der Arbeitsplatz und Ausrüstung zur Verfügung gestellt hat, bin ich zu grossem Dank verpflichtet.

Ich sage auch Herrn Fil. Lic. Göran Bergson für seine Assistenz bei der Infrarotspektroskopie Dank.

Ich bin auch dem *Schwedischen Naturwissenschaftlichen Forschungsrat* und *ASEA*, Västerås, sehr dankbar für ihre Unterstützung, ohne die sich diese Arbeit nicht hätte ausführen lassen.

Chemisches Institut der Universität Uppsala.

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Tryckt den 7 december 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB

New syntheses of 2,2'- and 3,3'-bithienyl

By SALO GRONOWITZ and HANS-OLOF KARLSSON

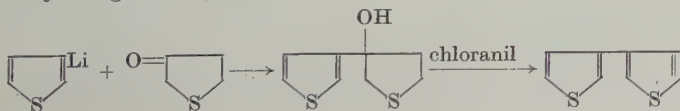
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New interest in the chemistry of terthienyl and bithienyl derivatives has been stimulated by the discovery that they are the nematocidal principles in *Tagetes erecta* [1, 2] and that bithienyl derivatives occur also in other Compositae [3]. Furthermore, the bithienyls enable the effect of the 2- and 3-thienyl groups in substitution reactions to be studied and also permit a comparative study of their stereochemistry with the biphenyls.

2,2'-Bithienyl has been prepared through the Ullmann syntheses with copper in the absence of solvent [4, 5]. Recently, Wynberg *et al.* [6] increased the yield by using dimethyl formamide as a diluent and in this way avoided the formation of polythienyls, although sometimes this reaction fails. Steinkopf *et al.* [7] prepared this compound from 2-thiophenemagnesium bromide and cupric chloride and found that, though it is obtained more rapidly, the yield is lower. However, lithium derivatives are often more readily available [8] and the recent paper by Tilak *et al.* [9] on the preparation of biaryls and bialkyls from organolithium compounds prompted the present authors to test this method for the preparation of bithienyls.

2-Thienyllithium, which is prepared in high yield through metalation of thiophene with butyllithium [10] (both compounds are commercially available) gave, with equimolar quantities of anhydrous cupric chloride, 2,2'-bithienyl in 54 % yield. Increasing the amount of cupric chloride or carrying out the reaction at -70° gave the same yield. As in the reaction between phenylmagnesium bromide and cupric chloride [11] a deficit of cupric chloride lowered the yield considerably, indicating that the role of the cupric chloride is not solely catalytic. When one-third of the molar quantity of cupric chloride was used the yield of 2,2'-bithienyl was only 25 %.

3,3'-Bithienyl has hitherto been very difficult to prepare in reasonable yields. It has been obtained in about 5 % yield in the reaction between phosphorous hexa- or heptasulphides and the sodium salt of butanetetracarboxylic acid [12, 13]. We could also confirm the result of Bantjes [13] that it is formed in very low yield (about 1 %) in the Ullmann reaction with 3-iodothiophene. One of the authors has previously suggested [8] that it should be possible to prepare 3,3'-bithienyl by reacting 3-ketotetrahydrothiophene with 3-thienyllithium followed by dehydration and aromatization of the tertiary alcohol, a method analogous to the elegant preparation of 2,3'-bithienyl by Wynberg *et al.* [14].



This method gave 3,3'-bithienyl in 17% yield when the intermediate alcohol or unsaturated compound was not isolated in a pure state but immediately aromatized with chloranil. However, we have not tried to modify this reaction in order to obtain higher yields, as we subsequently found that we could obtain 3,3'-bithienyl directly in 49% yield from 3-thienyllithium and cupric chloride. Through this reaction 3,3'-bithienyl has now become a readily available compound.

Experimental

2,2'-Bithienyl

An ethereal solution of 2-thienyllithium [10] was prepared in the usual nitrogen-swept four-necked apparatus, by adding at the refluxing rate 400 ml of 0.94 *N* *n*-butyllithium to 30.0 g (0.36 moles) of thiophene in absolute ether. After refluxing for 2 hr, the solution was allowed to cool and pressed with nitrogen into a stirred ice-cold suspension of 50 g (0.37 moles) of anhydrous cupric chloride in 150 ml of anhydrous ether. The reaction mixture was then refluxed for 3 hr, cooled in an ice-bath and decomposed by the careful addition of 300 ml of ice-cold 2 *N* hydrochloric acid. The ether phase was separated, washed with water, *N*-sodium carbonate solution and water. The ether was distilled off, the residue steam-distilled, extracted with ether, dried over calcium chloride and fractionated *in vacuo*. 16.0 g (54%) of 2,2'-bithienyl, b.p. 141–142°/24 mm Hg, m.p. 31–32° was obtained. It was identical with an authentic sample prepared through the Ullmann reaction with 2-iodothiophene [6], the identity being established by mixed m.p. and IR-spectrum (Fig. 1) determination.

3,3'-Bithienyl

Method 1.—To a stirred solution of 3-thienyllithium [15], prepared in the usual way at –70° from 21.2 g (0.13 moles) of 3-bromothiophene [16] in 50 ml ether and 125 ml of ethereal 1.13 *N* *n*-butyllithium, was added drop-wise 13.0 g (0.13 moles) of 3-keto-tetrahydrothiophene [14] in 10 ml of ether in the usual nitrogen-swept four-necked apparatus. Stirring was continued at –70° for 1 hr. The reaction mixture was then allowed to warm up and poured into 175 ml of ice-cold 6 *N* hydrochloric acid. The ether phase was separated and the aqueous phase extracted with ether. The combined ether layers were washed with water and dried over magnesium sulphate. The ether and *n*-butyl bromide was distilled off *in vacuo* leaving a yellow oil to which a hot solution of 35 g (0.14 moles) of chloranil in 200 ml of ethylene glycol was added. The mixture was refluxed for 1 hr, cooled and made alkaline with 200 ml of 2 *N* sodium hydroxide solution. Steam distillation gave 3.6 g (17%) of crude 3,3'-bithienyl, m.p. 115–125°. One recrystallization from ligroin (b.p. 80–100°) gave 2.8 g of 3,3'-bithienyl, m.p. 130–132°.

3,3'-Bithienyl

Method 2.—A solution of 3-thienyllithium, prepared as above from 100 g (0.61 moles) of 3-bromothiophene in 120 ml of absolute ether and 740 ml of 0.91 *N* *n*-butyllithium was pressed with nitrogen into an ice-cooled vigorously stirred suspension of 85 g (0.63 moles) of anhydrous cupric chloride in 225 ml of absolute ether. After refluxing for 3 hr, the mixture was cooled in an ice-bath and decomposed with 500 ml of ice-cold 2 *N* hydrochloric acid. The ether phase was worked up as in the synthesis

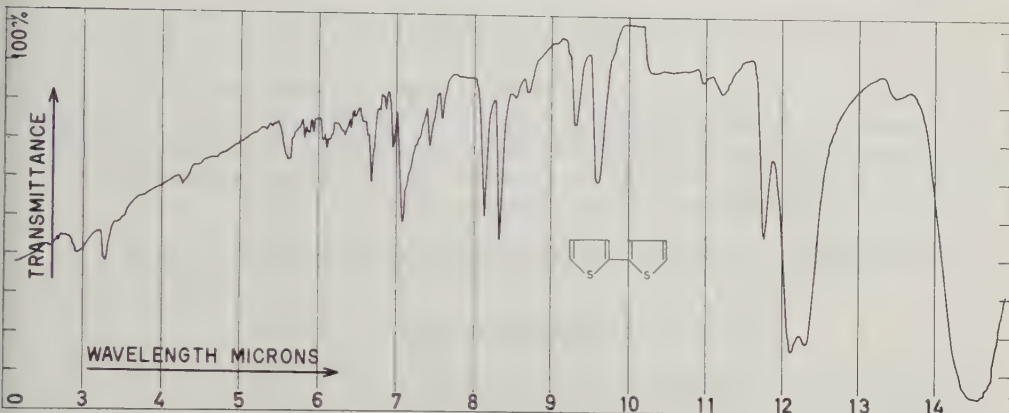


Fig. 1. IR-spectrum of 2,2'-bithienyl in KBr.

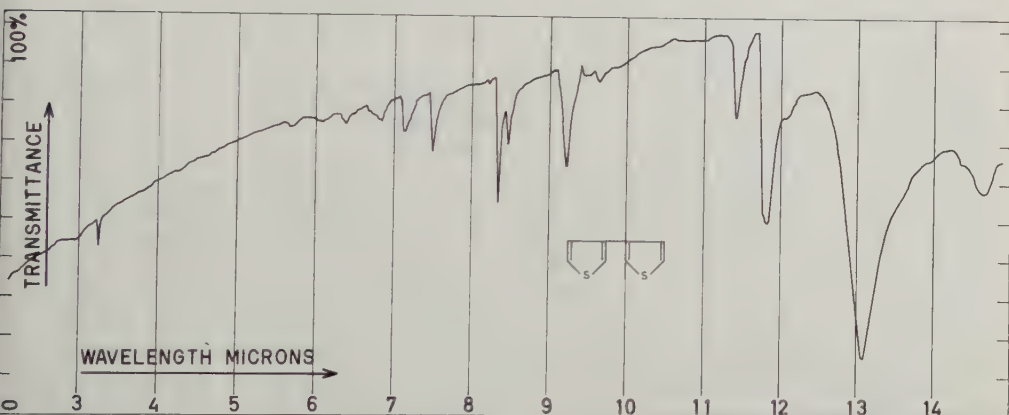


Fig. 2. IR-spectrum of 3,3'-bithienyl in KBr.

of 2,2'-bithienyl. Steam-distillation gave 25.1 g (49 %) of crude 3,3'-bithienyl, m.p. 108–120°. One recrystallization from ligroin (b.p. 80–100°) gave 18.6 g of pure 3,3'-bithienyl, m.p. 132–134°. It gave no m.p. depression with the specimen prepared above and had the same IR-peaks as those given in [17]. The characteristic peaks of 2,2'- and 2,3'-bithienyl [17] are absent in its IR-spectrum (fig. 2).

Anal. $C_8H_6S_2$ (166.2)

Calc.	C 57.79	H 3.64	S 38.57
Found	C 57.93	H 3.64	S 38.63

SUMMARY

2,2'- and 3,3'-bithienyl have been prepared in 50 % yield from 2- and 3-thienyllithium and cupric chloride. Through this method 3,3'-bithienyl, a hitherto almost unknown and difficultly accessible compound, is made easily available. 3,3'-Bithienyl has also been obtained through the reaction of 3-thienyllithium with 3-ketotetrahydrothiophene followed by aromatization with chloranil of the unsaturated intermediate.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Prof. Arne Fredga for his interest in this work and for all facilities put at their disposal as well as to Prof. Hans Wynberg for interesting discussions on the chemistry of bithienyls. The elementary analyses were carried out at the Analytical Department of this Institute. A grant from "*reservationsanslaget till biträdeshjälp m.m. åt docent*" to S.G. is gratefully acknowledged.

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Tryckt den 7 december 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB

Synthetic estrogenic isoflavonoids

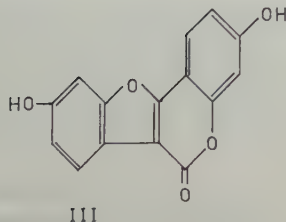
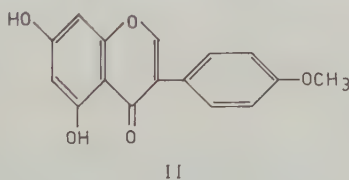
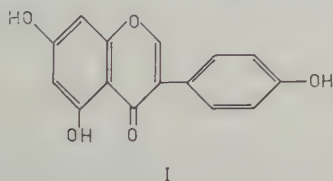
I. The synthesis of 3-(2'-thienyl)-5,7-dihydroxychromone

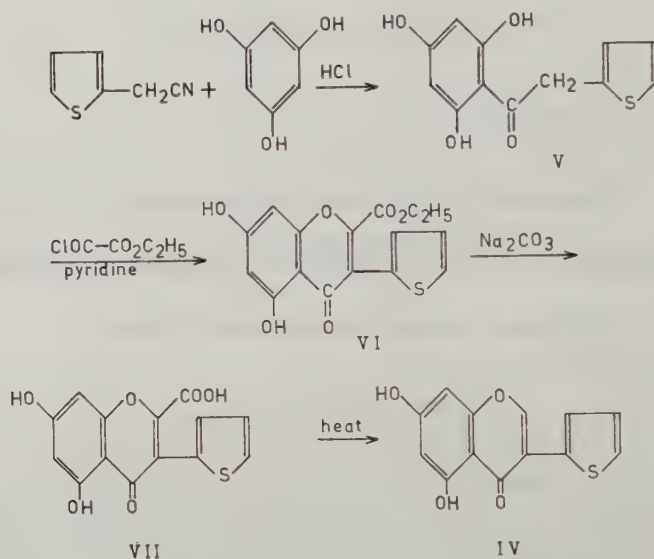
By SALO GRONOWITZ and ROLF EKMAN

With 4 figures in the text

In 1951, Bradbury & White [1] isolated the isoflavone genistein (I) and later Pople *et al.* [2] isolated genistein 4-methyl ether (biochanin A) (II) from subterranean clover (*Trifolium subterraneum* L.). They found that these compounds showed estrogenic activity and suggested that they were the active factors in this plant, found by Bennetts *et al.* [3, 4] to be responsible for the serious losses to the sheep-breeding industry in Western Australia. Later these and other estrogenically active isoflavones have been isolated also from red clover (*Trifolium pratense*) [2]. Coumestrol, a more strongly estrogenic compound, has recently been isolated from ladino clover (*Trifolium repens*) [5] and has been shown to be 3,9-dihydroxy-6H-benzofuro [3,2-c] [1] benzo-pyran-6-one, III [6, 7].

Many synthetic and naturally occurring isoflavones have been found to be estrogenic [8, 9, 10], and the influence of nuclear substituents and O-methylation has been investigated [10]. The effects of more drastic changes in the structure of the isoflavones, leading to isoflavonones, isoflavanols and isoflavens have likewise been investigated [10, 11]. The last-mentioned group of compounds, whose structure is





closely related to the stilbene estrogens, show especially high estrogen activity [10]. The influence of the isoflavones on the fertility and growth of mice has been especially investigated [12, 13, 14]. A comprehensive review on estrogens and related substances in plants by Bradbury & White [15] has appeared in 1954, where references to other papers on the estrogenic activity of isoflavones can be found.

It has been demonstrated in several instances that a replacement of a phenyl group by a thienyl group in a biologically active compound may result in enhanced activity, as has been found in the antihistamines, antispasmodics [16] and analgesics [17]. However, the opposite effect may sometimes be achieved, the thiophene isoster acting as inhibitor to the physiologically active phenyl analog [18, 19]. In the field of the estrogens, thiophene analogs of such estrogenic steroid hormones as 3-desoxy-isoequilenin [20], 3-desoxyequilenin [21] and isoestradiol [22] have been investigated. The thiophene analogs of estrogenic stilbenes have attracted special interest, as they are synthetically much more readily accessible [23, 24]. They have been extensively studied by Buu-Hoi *et al.* [25–28], who showed that they possess interesting biological properties.

As the first analog to estrogenic isoflavones, the authors have prepared 3-(2'-thienyl)-5,7-dihydroxychromone, IV. This compound is also of interest as it has been shown in the stilbestrol series [23] that the 2-thienyl group is more active than a phenyl group.

Recently, zinc cyanide [29] and formamide [30] have been used as sources for the $C_{(2)}$ atom of isoflavones in their synthesis from desoxybenzoins. Due to the lack of success with the first method in an attempted synthesis of Biochanin A and due to the latter method necessitating protection of all hydroxyl groups except that participating in the cyclization we used the excellent ethoxalyl chloride process of Baker *et al.* [31].

The Hoesch reaction between 2-thienyl cyanide and phloroglucinol gave a 67% yield of 2,4,6-trihydroxyphenyl-2-thienyl ketone V, showing that in this synthesis the

reactive 5-position of 2-thenyl cyanide does not cause any side-reactions. 2-Thenyl cyanide was prepared from 2-thenyl chloride and sodium cyanide using anhydrous acetone as the solvent as this, according to [32], prevents the formation of isocyanides. The unsuitability of alcohol as a solvent, due to the formation of ethyl thenylether, has been pointed out by Pettersson [33]. The thenyl chloride was prepared through chloromethylation of thiophene according to a modification by Fredga & Palm [34].

The ring closure to the chromone through ethoxalylolation was carried out according to a more rapid modification of the Baker synthesis by Yoder *et al.* [35] and gave 2-carbethoxy-3-(2'-thienyl)-5,7-dihydroxychromone VI, m.p. 192–193° in 74 % yield. Of the two different methods used by Baker *et al.* [31] for the hydrolyses of the carbethoxy isoflavones, the more rapid one using sodium carbonate in aqueous acetone solution was the preferred one and gave 2-carboxy-3-(2'-thienyl)-5,7-dihydroxychromone VII in 77 % yield. The acid crystallised in yellow needles with one molecule of water of crystallization when recrystallized from aqueous ethanol, m.p. 258–260°. It could be obtained anhydrous by heating *in vacuo* over phosphorous pentoxide at 120°.

Decarboxylation by heating at just above its melting-point in small portions gave IV in high yield. When recrystallized from aqueous ethanol, IV was obtained with one molecule of water of crystallization.

The biological tests were carried out by Dr. Anna Nilsson at the Royal Agricultural College and will be reported elsewhere.

Experimental

2-Thenyl cyanide

2-Thenyl chloride was prepared from 40 g (0.48 moles) of thiophene, 15 g paraformaldehyde and hydrogen chloride, according to the description of Fredga & Palm [34]. The undistilled 2-thenyl chloride was refluxed with vigorous stirring for 20 hr with 25 g of sodium cyanide and 3 g of sodium iodide in 100 ml of anhydrous acetone. After cooling the precipitate was filtered off, the acetone distilled off and the residue taken up in benzene and washed twice with hot water. The benzene phase was dried with sodium sulphate and fractionated, giving 27 g (46 %) of 2-thenyl cyanide, b.p. 115–118°/22 mm Hg.

2,4,6-Trihydroxyphenyl-2-thenyl ketone, V

A solution of 9.6 g (0.078 moles) of 2-thenyl cyanide and 9.8 g (0.078 moles) of anhydrous phloroglucinol in 80 ml of anhydrous ether was saturated with dry hydrogen chloride at 0°. The reaction mixture was stored at the same temperature for 24 hr and then again saturated with hydrogen chloride at 0°. After additional 24 hr at 0°, the ether was decanted off, the cake of ketiminhydrochloride was washed with ice-cold ether and then hydrolyzed by refluxing for 1 hr with 400 ml of 2 % hydrochloric acid. After cooling, 13 g (67 %) of ketone crystallized out as brownish needles. M.p. 132–135°, after recrystallization from 50 % ethanol and drying over phosphorus pentoxide (IR-spectrum, Fig. 1).

Anal. C₁₂H₁₀O₄S (250.2)

Calc.	C 57.60	H 4.03	S 12.81
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Found	C 57.61	H 4.04	S 12.56
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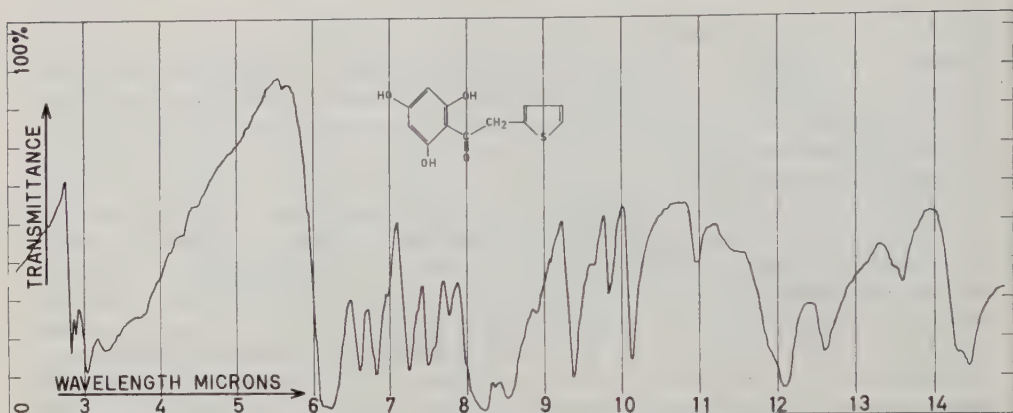


Fig. 1. IR-spectrum of 2,4,6-trihydroxy-phenyl-2-thienyl ketone in KBr.

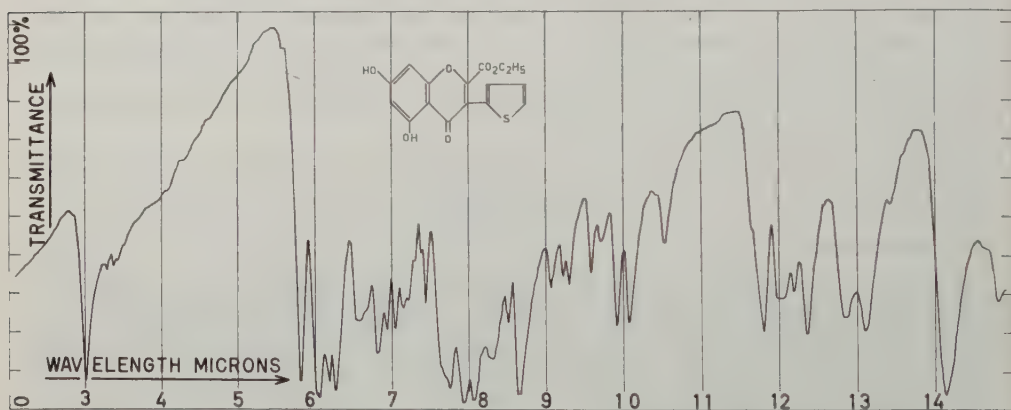


Fig. 2. IR-spectrum of 2-carbethoxy-3-(2'-thienyl)-5,7-dihydroxychromone in KBr.

2-Carbethoxy-3-(2'-thienyl)-5,7-dihydroxychromone, VI

To an ice-cooled solution of 5.0 g (0.020 moles) of V in 28 ml of anhydrous pyridine was added 11 ml (0.08 moles) of ethoxalyl chloride [36]¹ at such a rate that the temperature did not rise above 25°. The reaction mixture was then heated to 65° for 20 min, cooled and poured into dilute hydrochloric acid. The aqueous phase was extracted with chloroform and the combined organic phases were washed with 10 % hydrochloric acid, dried over magnesium sulphate and the chloroform removed *in vacuo*. The crystalline residue was recrystallized from 50 % ethanol, yielding 4.9 g (74 %) of the desired yellow ester, m.p. 192–193° (IR-spectrum, Fig. 2).

Anal. $C_{16}H_{12}O_6S$ (332.3)

Calc.	C 57.82	H 3.64	S 9.65
Found	C 57.53	H 3.67	S 9.62

¹ We obtained more reproducible yields of ethoxalyl chloride and better results in the ethoxalyl-ation reaction with ethoxalyl chloride prepared according to [36] than when prepared according to [37].

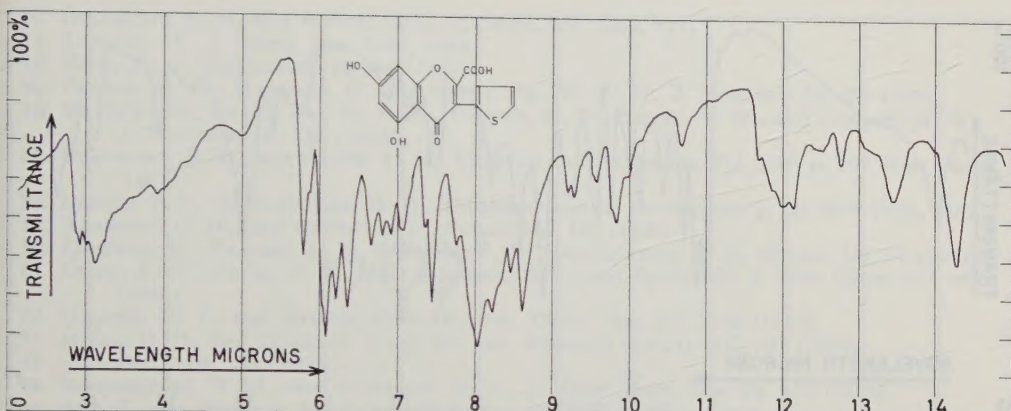


Fig. 3. IR-spectrum of 2-carboxy-3-(2'-thienyl)-5,7-dihydroxychromone in KBr.

2-Carboxy-3-(2'-thienyl)-5,7-dihydroxychromone, VII

2.0 g (0.0060 moles) of VI in 30 ml of acetone and 30 ml of 5 % aqueous sodium carbonate was refluxed for 4 hr. The acetone was distilled off. The solution was cooled and carefully acidified with 2 *N* hydrochloric acid to pH 3. The acid was filtered off and recrystallized from aqueous ethanol, yielding 1.5 g (77 %) of VII, which crystallized in yellow needles with 1 mole of water.

Anal. $C_{14}H_8O_6S \cdot H_2O$ (322.2)

Calc. C 52.18 H 3.13 S 9.93

Found C 52.25 H 3.15 S 9.90

After drying *in vacuo* over phosphorus pentoxide at 120°, anhydrous VII, m.p. 258–260° dec. was obtained (IR-spectrum, Fig. 3).

Anal. $C_{14}H_8O_6S$ (304.3)

Calc. C 55.24 H 2.65 S 10.53

Found C 54.95 H 2.77 S 10.46

3-(2'-Thienyl)-5,7-dihydroxychromone, IV

1.0 g of VII was heated in a Pyrex test tube to 265° in an oil bath for 10 min until the evolution of carbon dioxide had ceased. After cooling, the melt was dissolved in the minimum amount of boiling ethanol and decolorized with charcoal. After addition of an equal volume of water, 0.8 g (86 %) of IV crystallized out in almost colorless needles, which contained 1 mole of crystal water.

Anal. $C_{13}H_8O_4S \cdot H_2O$ (278.3)

Calc. C 56.11 H 3.62 S 11.52

Found C 56.00 H 3.38 S 11.25

After drying at 120° *in vacuo* over phosphorus pentoxide, anhydrous IV, m.p. 169–170° was obtained (IR-spectrum, Fig. 4).

Anal. $C_{13}H_8O_4S$ (260.3)

Calc. C 59.99 H 3.10 S 12.32

Found C 59.81 H 3.15 S 12.22

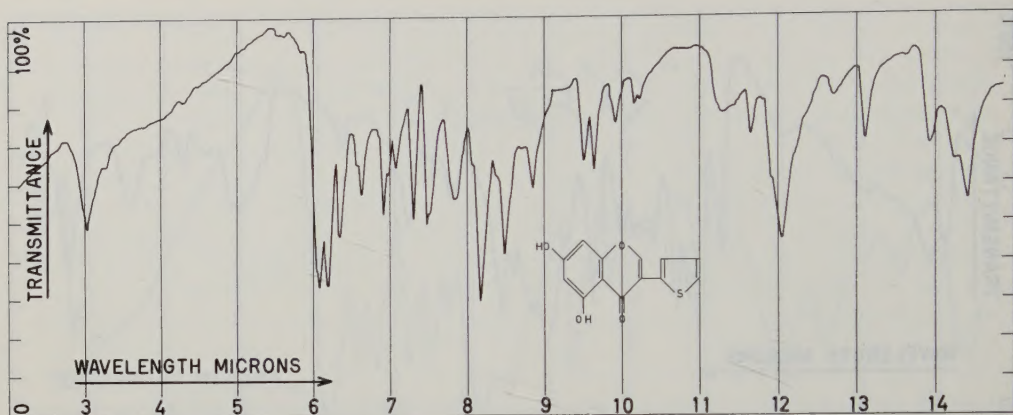


Fig. 4. IR-spectrum of 3-(2'-thienyl)-5,7-dihydroxychromone in KBr.

A Perkin Elmer model 21 spectrophotometer equipped with a sodium chloride prism was used for the IR-measurements. The melting points were determined in a hot-stage microscope.

SUMMARY

3-(2'-thienyl)-5,7-dihydroxychromone has been prepared as an analog to naturally occurring estrogenic isoflavones. It has been prepared through a Hoesch synthesis from 2-thienyl cyanide and phloroglucinol, followed by cyclization of the desoxybenzoin with ethoxalyl chloride.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Prof. Arne Fredga for all facilities put at their disposal. Thanks are also due to Mr. S. Johansson for recording the IR-spectra. The elementary analyses were carried out at the Analytical Department of this Institute. A grant from "*reservationsanslaget till biträdes hjälp m.m. åt docent*" is gratefully acknowledged.

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Tryckt den 7 december 1960

Uppsala 1960. Almqvist & Wiksells Boktryckeri AB

